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In the right place and at the right time: cellular and functional neuroanatomy of endocannabinoids and neuropeptides

Joanne Bakker

Karolinska Institutet

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In the right place and at the right time: cellular and functional neuroanatomy of endocannabinoids and neuropeptides

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By

Joanne Bakker

Principal Supervisor:
Tibor Harkany
Karolinska Institutet
Department of Neuroscience

Co-supervisors:
Tomas Hökfelt
Karolinska Institutet
Department of Neuroscience

Konstantinos Meletis
Karolinska Institutet
Department of Neuroscience

Opponent:
Mike Ludwig
The University of Edinburgh
Center for Discovery Brain Sciences

Examination Board:
Malin Lagerström
Uppsala University
Department of Neuroscience

Dick Nässel
Stockholm University
Department of Zoology

Eva Hedlund
Stockholm University
Department of Biochemistry and Biophysics

“Biology gives you a brain. Life turns it into a mind.”

-Jeffrey Eugenides,

‘Middlesex’

ABSTRACT

A plethora of chemical signals, including endocannabinoids and neuropeptides, enable neuronal communication beyond the classical neurotransmitters. Many of these molecules are also expressed during (early) brain development, helping neurons to mature and neuronal circuits to take form. This thesis focuses on the roles of these "unusual transmitters" in (the establishment of) brain circuits.

Besides neurons, the brain also consists of glial cells: astrocytes, microglia, and oligodendrocytes. Glial cells play invaluable roles in not only the adult brain, but also in the developing nervous system. Endocannabinoid signaling promotes several processes in neuronal development, including axonal growth and elongation mediated by the growth cone. In **study I**, we inhibited the degrading enzyme of the endocannabinoid 2-arachidonoylglycerol (2-AG), which resulted in errors in axonal fasciculation and premature maturation of oligodendrocytes within axonal bundles. Neuronal growth cones were repulsed via interactions of the axon guidance cues Slit and Robo. Thus, cannabinoid type 1 receptor (CB_1R) signaling repositioned Robo1 to neuronal growth cones on the one hand, while CB_2R signaling induced excess production of Slit2 in oligodendrocytes on the other. Taken together, this indicates that endocannabinoids regulate downstream Slit/Robo signaling in embryonic development.

In the first two postnatal weeks, the newly established brain circuits undergo extensive remodeling. In **study II**, we discovered that the neuropeptide galanin is transiently expressed in cells in the somatosensory thalamus during this time, which was confirmed in the galanin-Cre::tdTomato mouse. We designed a 3D-printed extension for the stereotactic apparatus to perform viral circuit tracing in 7-day old pups, at a time of active Cre expression. We established that the cells that transiently express galanin are neurons in the ventrobasal nucleus of the thalamus (VB) projecting towards the somatosensory cortex. The robust expression of galanin in VB soma and the discovery of galanin receptors in the VB hints at potential somatodendritic release of galanin, which could be involved in the postnatal refinement of the VB.

A brain region known for its diverse expression of neuropeptides, including galanin, is the hypothalamus. In **study III**, we attempted to analyze the molecular diversity of the hypothalamus using single-cell sequencing. This resulted in 62 neuronal clusters segregated by enzymes involved in the turnover of neurotransmitters and by neuropeptides. Using amongst others viral circuit tracing, we investigated how hypothalamic neurons functionally integrate in hitherto unknown brain circuits. In **study III**, we could place a novel dopaminergic neuronal subtype in a brain circuit receiving neuropeptidergic inputs from the suprachiasmatic nucleus and projecting towards the median eminence. These neurons are probably involved in the circadian control of release of dopamine which controls the secretion of prolactin. In **study IV**, we uncovered a new neuronal stress circuitry

increasing cortical alertness. It links stress-responsive hypothalamic neurons via volume transmission of the neurotrophin ciliary neurotrophic factor (CNTF) in the cerebrospinal fluid to the noradrenergic neurons in the pontine locus coeruleus. These neurons in turn project to the prefrontal cortex inducing long-lasting excitability in response to acute stress.

LIST OF SCIENTIFIC PAPERS

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LIST OF ABBREVIATIONS

ABHD	α-β-hydrolase domain
ACTH	Adrenocorticotropic hormone
AEA	N-arachidonoyl ethanolamine, “anandamide”
2-AG	2-arachidonoylglycerol
BAC	Bacterial artificial chromosome
CAM	Cell adhesion molecule
CB_1R	Cannabinoid receptor type 1
CB_2R	Cannabinoid receptor type 2
CF	Climbing fiber
CGE	Caudal ganglionic eminence
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CNTFR	Ciliary neurotrophic factor receptor
CP	Cortical plate
Cre	Cre recombinase
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
CTA	Corticothalamic afferent
DAT	Dopamine transporter
DCN	Dorsal column nuclei
DAGL	Diacylglycerol lipase
dLGN	Dorsal lateral geniculate nucleus
DREADD	Designer receptor activated by designer drugs
DRG	Dorsal root ganglion
DTB	Diencephalon-telencephalon border
E	Embryonic day
(E)GFP	(Enhanced) green fluorescent protein
Erk	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
GABA	Gamma-aminobutyric acid

Gal	Galanin
GalR	Galanin receptor
GALP	Galanin-like peptide
Gbx2	Gastrulation Brain Homeobox 2
GCaMP	Genetically encoded calcium indicator
GENSAT	Gene Expression Nervous System Atlas
GIRK	G protein-gated inwardly rectifying potassium channel
GMAP	Galanin message-associated peptide
GPCR	G protein-coupled receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
Icv	Intracerebroventricular
iDISCO^{+}	**I**mmunolabeled-enabled three-**d**imensional **i**maging of **s**olvent-**c**leared **o**rgans
IZ	Intermediate zone
KO	Knockout
KOMP	Knockout mouse project
LC	Locus coeruleus
LDCV	Large dense core vesicle
LGE	Lateral ganglionic eminence
LIF	Leukemia inhibitory factor
MAPK	Mitogen-associated protein kinase
MBP	Myelin basic protein
MGE	Medial ganglionic eminence
MGL	Monoacylglyercol lipase
MZ	Marginal zone
NAE	N-acylethanolamine
NAPE	N-acyl-phosphatidylethanolamine
NGF	Nerve growth factor
NPC	Neural progenitor cell
NPY	Neuropeptide Y
OPC	Oligodendrocyte precursor cell

P	Postnatal day
p	Prosomere
PC	Purkinje cell
PeVN	Periventricular nucleus of the hypothalamus
PFC	Prefrontal cortex
PKC	Protein kinase C
PLD	Phospholipase D
PNS	Peripheral nervous system
POMC	Pro-opiomelanocortin
PP	Preplate
Pr5	Principal sensory trigeminal nucleus
PrV2	Ventral part of Pr5
PrV3	Dorsal part of Pr5
r	Rhombomere
rAAV	Recombinant adeno-associated virus
Robo receptor	Roundabout receptor
Scgn	Secretagogin
SP	Subplate
Sp5	Spinal trigeminal nucleus
SVZ	Subventricular zone
TCA	Thalamocortical afferent
TeLC	Tetanus toxin-light chain
TG	Trigeminal ganglion
TH	Tyrosine hydroxylase
Δ^9-THC	Δ^9-tetrahydrocannabinol
VAChT	Vesicular acetylcholine transporter
VB	Ventrobasal nucleus of the thalamus
VGLUT	Vesicular glutamate transporter
VPL	Ventroposterolateral nucleus of the thalamus
VPM	Ventroposteromedial nucleus of the thalamus
VZ	Ventricular zone

1 INTRODUCTION

Neurons can communicate with each other in different ways. They can be directly connected through electrical synapses or use a plethora of signaling molecules at chemical synapses. These chemical messengers range from classical amino acid neurotransmitters, monoamines **(study III, IV)**, hormones, and neuropeptides **(study II, III)** to lipids (e.g., endocannabinoids, **study I**), and gases such as nitric oxide. The variety of these communication signals and the distance they can travel are astonishing – they can whisper, talk, scream and shout to their target cells located close by, or in the other end of the brain. This work deals with unusual forms of neuronal signals beyond the classical amino acid and monoamine neurotransmitters, and how they help shape the mouse brain and its neuronal circuits.

I will firstly discuss the basic processes of mouse brain development, which are sensitive to the actions of endocannabinoids **(study I)** and/or the neuropeptide galanin **(study II)**, which I will subsequently introduce. Finally, the organization and circuits of the hypothalamus **(study III and IV)** will be discussed.

1.1 OVERVIEW OF BRAIN DEVELOPMENT

Our understanding of the organization of the brain is helped by the study of its development. I will briefly discuss the development of the laminated structure of the cortex, which has been studied in most detail and is relevant for **study I**. As an example of how neural circuits are formed, I will consider the development of the somatosensory circuit and particularly the somatosensory thalamus, which is the region of interest in **study II**.

1.1.1 Gastrulation and neurulation

The first critical event of development is **gastrulation**, when the primary germ layers (endoderm, mesoderm, and ectoderm) are formed. Next, the transient structure of the notochord induces **neurulation**, during which the neuroectoderm lying directly above it changes from a neural plate into a neural tube. The multipotent neural stem cells in this structure give rise to both the brain and the spinal cord, while adjacent neural crest cells generate (amongst others) the peripheral nervous system (PNS). In the developing brain, the proliferation of cells induces bulges in the neural tube, which distinguish three primary vesicles: prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon

(hindbrain). The forebrain later splits to become telencephalon and diencephalon, and the hindbrain segments into the metencephalon and the myelencephalon. The telencephalon ultimately gives rise to the cortex, the striatum, the limbic system, and the olfactory bulb. The diencephalon develops into the thalamus and hypothalamus. All these processes are influenced by (combinations of) gradients of morphogens such as Wnt, Sonic hedgehog, and fibroblast growth factors, which are secreted by organizing centers and induce the expression of transcription factors in the responsive tissue (Erzurumlu and Gaspar, 2012).

In the embryonic brain of most vertebrates, three cavities filled with fluid are initially formed by the bending of the hollow neural tube: one in the forebrain, one in the midbrain and one in the hindbrain (Lowery and Sive, 2009). The forebrain vesicle later splits into two lateral vesicles and the third ventricle in the midline; the midbrain ventricle turns into the cerebral aqueduct connecting the third and fourth ventricle; and the cavity in the hindbrain becomes the fourth ventricle (Lowery and Sive, 2009).

1.1.2 Proliferation and migration of neurons

The **proliferation** of precursor cells takes place in the ventricular zone (VZ), the innermost cell layer surrounding the lumen of the neural tube. In the first phase of proliferation progenitor cells expand through symmetrical division. In the second phase asymmetrical division gives rise to a new progenitor cell and a postmitotic (daughter cell) neuron, which will migrate to its final destination. In the developing cortex immature pyramidal cells climb from the proliferative zones along radial glia during **radial migration**. This process occurs in an inside-out manner: first-born neurons migrate to the inner layers of the cortex, whereas later-born neurons travel to the outer layers of the cortex (Rakic, 2000). During **tangential migration** immature interneurons move parallel to the brain surface to their target region from a second proliferative zone in the ventral telencephalon. This other proliferative zone consists of the medial, lateral and caudal ganglionic eminences (respectively MGE, LGE and CGE) and later develops into the basal ganglia (Gelman and Marín, 2010).

All these migratory events change the structure of the developing cortex (see **figure 1**). The intermediate zone (IZ) emerges above the VZ. Superficially to the intermediate zone, the preplate (PP) appears, the first layer to receive neurons through migration around embryonic day (E) 11. The subventricular zone (SVZ) emerges between the ventricular and intermediate zones. As the VZ becomes depleted of cells in the first wave of migration, most cells migrate from the SVZ in the second wave. This next wave of migrating neurons between E13 and E14 splits the PP into two regions, the marginal zone (MZ) and the subplate (SP). Between them the cortical plate (CP) is formed, later developing into the six layers of the cerebral cortex. The MZ, SP, VZ and SVZ are all transient structures in the embryonic brain that are absent from the adult brain. The IZ ultimately becomes white matter.

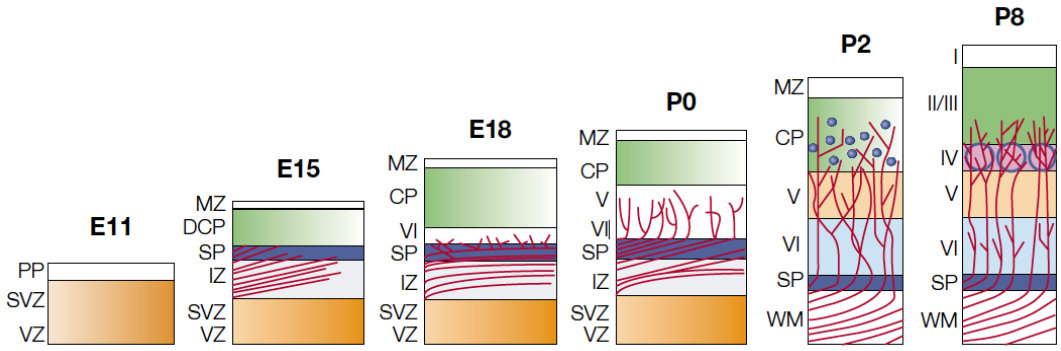


Figure 1. Cortical lamination and ingrowth of thalamocortical afferents. *At embryonic day (E) 11, the first postmitotic cells form the preplate (PP), which is split into the marginal zone (later layer I) and the subplate (SP) by the arrival of neurons in the cortical plate (CP). Thalamic axons (red lines) arriving at E15 accumulate in the SP. Around postnatal day (P) 0, most thalamic fibers invade the CP and layers V and VI, forming their barrel pattern around cortical layer IV neurons from P2. SVZ = subventricular zone, VZ = ventricular zone, MZ = marginal zone, DCP = deep part of the cortical plate, IZ = intermediate zone, I-VI = layer of cortex, WM = white matter. Reproduced from (Lopez-Bendito and Molnar, 2003).*

Although we know many of the molecular mechanisms underlying cortical development, less is known about the patterning of non-cortical areas which do not have a laminated structure. The thalamus consists, for example, almost exclusively of glutamatergic projection neurons organized in several different nuclei, each with their own typical connectivity pattern and functional role (Evangelio et al., 2018; Gezelius and López-Bendito, 2017).

Analogous to the organization of the hindbrain (rhombencephalon) in transverse segments called "rhombomeres" based on morphological characteristics, specific patterns of gene expression, and boundaries restricting cell migration between rhombomeres, it was suggested that the forebrain (prosencephalon) can be divided into "prosomeres" (Bulfone et al., 1993; Rubenstein et al., 1994). The most rostral prosomere (p3) forms the prethalamus, while p2 is the origin of the thalamus and the epithalamus/habenula (Gezelius and López-Bendito, 2017).

Thalamic neurons are born between E10-E15, at which time the different nuclei of the thalamus are not easily distinguished (Gezelius and Lopez-Bendito, 2017). Thalamic nuclei seem to be populated by lineage-derived clones of thalamic radial glia that occupy a few nuclei after ongoing radial or tangential migration (Shi et al., 2017), mostly defined by their rostro-caudal and dorso-ventral position (Wong et al., 2018). In other words, each thalamic radial glia cell is partially specified for producing neurons that populate a subset of thalamic nuclei (Nakagawa, 2019).

The molecular mechanisms guiding specification and differentiation of specific thalamic nuclei are still predominantly unknown (Suzuki-Hirano et al., 2011). Neurons in all thalamic nuclei express the transcription factor Gastrulation Brain Homeobox 2 (*Gbx2)*, but the onset and temporal window differ between nuclei (Chen et al., 2009; Jones and Rubenstein, 2004). The expression of *Gbx2* might therefore contribute to the distinction between the thalamus and surrounding structures (Mallika et al., 2015), but also to the segregation of different thalamic nuclei, possibly by regulating an extracellular signaling

pathway (Chen et al., 2009). Indeed, some genes in the thalamus are region-specific and sometimes even nucleus-specific during certain stages of development, although most genes undergo dynamic changes during development (Suzuki-Hirano et al., 2011; Yuge et al., 2011).

1.1.3 Gliogenesis

Around birth the balance shifts from neurogenesis to **gliogenesis** (see **figure 2**), although proliferation and migration of glial precursors already starts before birth. Glial progenitors proliferate in the subventricular zone and migrate to other brain regions, where they differentiate into astrocytes and oligodendrocytes. In mammals, the multiciliated **ependymal cells** lining the ventricles are derived from embryonic radial glia cells (Redmond et al., 2019) and appear during late prenatal and early postnatal stages (Spassky et al., 2005).

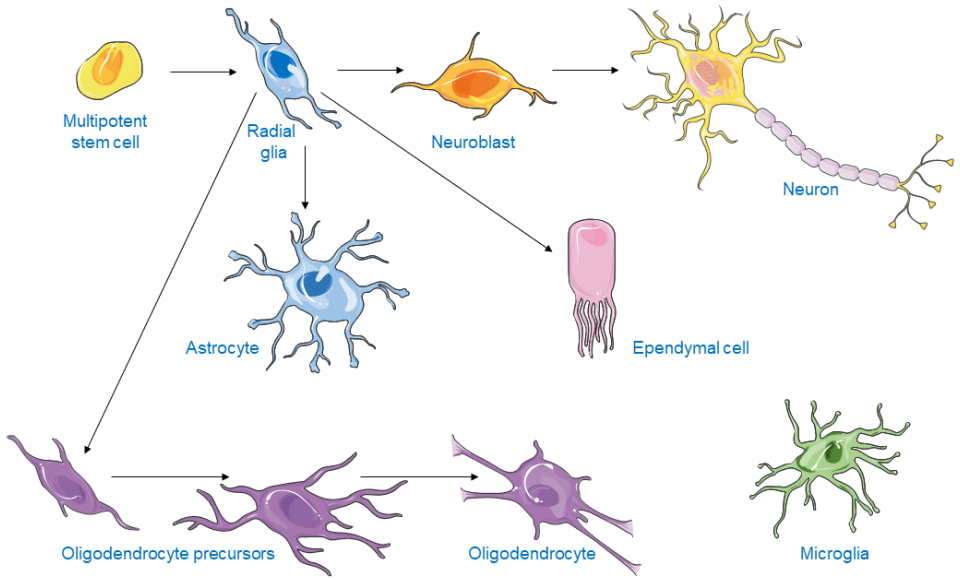


Figure 2. The differentiation of the brain cells. *Figure adapted from an illustration by Smart Servier Medical Art, https://smart.servier.com, CC BY 3.0.*

The glial progenitor cells that give rise to myelinating **oligodendrocytes** are called oligodendrocyte precursor cells (OPCs). In the mouse, OPCs are generated in three sequential waves from different parts of the telencephalic VZ: 1) from precursors in the MGE starting at E12.5; 2) from LGE/CGE precursors starting at E15.5; and 3) from cortical precursors starting around postnatal day (P) 0 (Kessaris et al., 2006). Intriguingly, these different populations are functionally redundant, and the first population (from MGE) is eliminated postnatally (Kessaris et al., 2006). A possible explanation is the marked transcriptional similarity of postnatal OPCs independent of their origin (Marques et al., 2018). Only two waves of OPC generation occur in the spinal cord: the first OPCs appear in the ventral VZ at around

E12.5, while the second wave starts in the dorsal cord at E15.5 (reviewed by (Bergles and Richardson, 2015)).

Shortly before birth some OPCs in the spinal cord start to produce differentiated oligodendrocytes that express myelin genes, including the myelin proteolipid protein and myelin basic protein (MBP) (Bergles and Richardson, 2015). In the rat brain the first myelinated axonal processes are visible from P4, while the last areas are starting to be myelinated at P14 (Downes and Mullins, 2014). OPCs persist in the postnatal central nervous system (CNS) and continue to divide and generate myelinating oligodendrocytes, although at a decreasing rate throughout adult life in rodents (Bergles and Richardson, 2015).

Astrogenesis starts at around E18 and lasts until at least P7 (see ((Reemst et al., 2016)). **Astrocytes** are distributed across the whole central nervous system and most likely migrate to their area of destination shortly after birth in the VZ or SVZ, as the distribution in the embryonic and adult brain has been reported to be similar (Reemst et al., 2016). Our understanding of how progenitor cells mature into the heterogenous cell populations of astrocytes is incomplete (Zhang and Barres, 2010), due to the lack of reliable and specific markers to define (immature) astrocytes throughout their development (Reemst et al., 2016). Astrocytes are mitotic cells and can thus continue to divide and differentiate, for example after injury (Reemst et al., 2016).

In contrast, **microglia** are thought to derive from primitive myeloid progenitor cells, which invade the mouse brain from E8 and gradually expand between E10.5-E14.5 (Swinnen et al., 2013). The second increase in the number of microglia, between E14.5-E15.5, is probably caused by the entry of new microglia progenitors from the periphery (Swinnen et al., 2013). Between E10-E12, the first microglia progenitors are observed in the meninges and within the lateral ventricles, but only a few can be found in the neuroepithelium (Swinnen et al., 2013). Microglia only start to invade the cortical plate around E17, while also becoming more ramified (Reemst et al., 2016).

Crucially, glial cells, including OPCs and oligodendrocytes, astrocytes and microglia, are not only essential for the proper functioning of the adult brain, but also influence the developmental processes discussed below, such as axonal outgrowth, synaptogenesis and synaptic pruning (reviewed by (Allen and Lyons, 2018; Reemst et al., 2016)).

1.1.4 Axonal elongation and pathfinding

Once developing neurons have reached their target region, they must get integrated into neuronal networks by connecting to other neurons with their axons and dendrites. **Axon growth** is mediated through the growth cone, a “specialized bulbous structure at the end of growing axons with dynamic

filamentous extensions called filopodia, sensing the environment and responding quickly to adhesion and guidance molecules" (Ypsilanti et al., 2010) which attract or repulse the growth cone. However, growth cones and axons do not move just because they sense chemical guidance signals: they still need to exert mechanical forces on their environment to be able to change their position in space (see (Franze, 2020)).

For that purpose, actin filaments in the growth cone are on the one hand cross-linked to the molecular motor protein myosin (Vicente-Manzanares et al., 2009) and can, on the other hand, be coupled to the extracellular environment by "point contacts" (Franze, 2020). These neuron-specific protein complexes serve as molecular clutches and include many adaptor and signal proteins, such as cell-adhesion molecules (CAMs) (Franze, 2020). Most guidance cues affect one or more of the Rho GTPases, which are important upstream regulators of actin polymerization and myosin activity in the growth cone (Hall and Lalli, 2010). The known guidance cues and their receptors are mostly conserved in model systems like the commissural axons of the spinal cord, thalamocortical axons, and axons in the corpus callosum and optic chiasm, and an overview can be found in **figure 3** (reviewed by (Stoeckli, 2018)). In addition to Slit/Robo signaling, to which we will return shortly, other examples include netrin (Métin et al., 1997; Richards et al., 1997) and semaphorins (Bagnard et al., 1998; Chédotal et al., 1998; Polleux et al., 1998).

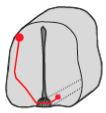
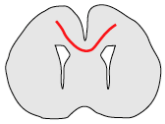
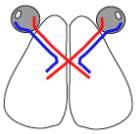
	Spinal cord	Brain		
				
Guidance cue/ receptor	**dl1 commissural axons**	**Thalamo-cortical axons**	**Corpus callosum**	**Optic chiasm**
Netrin/Dcc	+	+	+	+
Slit/Robo	+	+	+	+
Sema3/neuropilin	+	+	+	+
Sema6/plexin	+	+	?	+
Ephrin/Eph	+	+	+	+
Wnt/Frz3 or Ryk	+	+	+	+
Draxin/Dcc	+	+	+	?

Figure 3. Axon guidance within the brain. *Most of the guidance cues discovered in the "simple" navigation of the dl1 commissural axons in the spinal cord are conserved, seeing that they are also used in the navigation of thalamo-cortical axons, of axons in the corpus callosum and of those forming the optic chiasm. Slit/Robo signaling is highlighted, as it will be discussed later. Sema = semaphorin. Table reproduced from (Stoeckli, 2018).*

The thalamocortical afferents (TCAs) cross the diencephalon-telencephalon border (DTB) by E12.5 and are included in the internal capsule at E13.5 (see **figure 4**). After reaching the subplate at E15, they wait there before reaching the cortical plate (P0) and forming the barrel pattern (P3-P5) in layer IV. So-called corridor cells as well as other neurons located *en route* provide guidance information to TCAs (see (López-Bendito, 2018)). Reciprocal connectivity of the thalamocortical circuit also depends on a transient cortical cell population in the subplate whose axons interact with growing TCAs, as postulated

in the "handshake hypothesis" (López-Bendito, 2018; Molnár and Blakemore, 1995). This hypothesis has been debated for decades, but recent evidence from studies using conditional knock-out models provides support (López-Bendito, 2018).

Growing axons can adhere to each other, potentially forming groups of axons known as "fascicles", which follow similar growth trajectories. Molecular interactions between proteins in the axonal membranes (axolemmas) can promote either fasciculation, defasciculation (when an axon leaves a bundle of fasciculated axons) or growth cone repulsion (Davis et al., 2017).

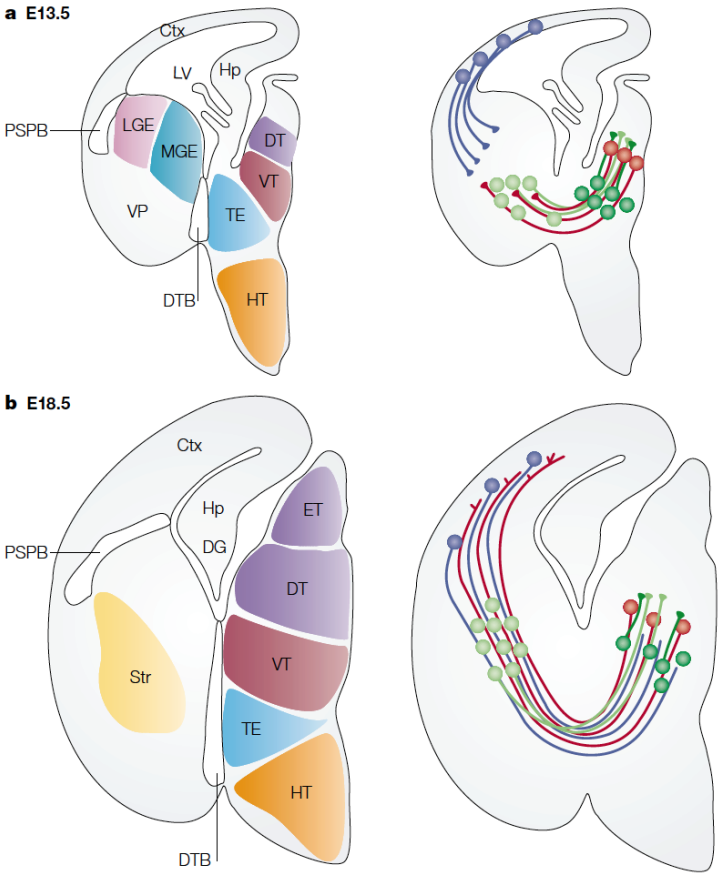


Figure 4. Growth of thalamocortical and corticothalamic axons during mouse forebrain development. *(A) Thalamic axons (red lines) have left the dorsal thalamus (DT) on embryonic day (E) 13.5. They descend between ventral thalamic cells (dark green) and cross the border between the diencephalon and telencephalon (DTB), passing cells in the internal capsule (light green) before extending through the medial and lateral ganglionic eminences (MGE and LGE). At E13.5, corticofugal axons (blue lines) leave from the cortex, pausing at the pallial-subpallial boundary (PSPB). (B) Both thalamocortical and corticofugal axons approach their targets at E18.5, with the thalamic axons mainly located at the intermediate zone and subplate of the cortex and the corticofugal axons arriving to the dorsal thalamus in a topographically organized manner. DG = dentate gyrus, ET = epithalamus, Hp = hippocampus, HT = hypothalamus, LV = lateral ventricle, Str = striatum, TE = thalamic eminence, VP = ventral pallium, VT = ventral thalamus. Image reproduced from (Lopez-Bendito and Molnar, 2003).*

1.1.4.1 Robo-Slit signaling

One example of guidance molecules mediating attraction/repulsion signals is the pair of Robo and Slit. The Roundabout (Robo) receptor was originally discovered in a mutation screen for defects in growth cone guidance in *Drosophila melanogaster* (Seeger et al., 1993). Subsequently, the secreted Slit proteins (one in invertebrates, three in vertebrates) were found to be the ligands of Robo in several species (Brose et al., 1999; Kidd et al., 1999).

In addition to the spinal cord, Slit and Robo are also expressed in the developing forebrain and mediate repulsion there (Nguyen Ba-Charvet et al., 1999). In the mouse forebrain Slit1 and Slit2 cooperate to fulfill their three functions in axonal pathfinding, namely (1) preventing longitudinal axonal tracts to

enter ventral regions; (2) preventing axonal extension toward and across the midline; and (3) guiding of axons into specific regions, such as commissures (López-Bendito et al., 2007). These effects are mediated by the axonal transmembrane receptors Robo1 and Robo2, which mostly have overlapping and redundant roles (López-Bendito et al., 2007). Slit/Robo signaling linking to adaptor proteins also plays a role in cell polarity, cell adhesion and cytoskeletal dynamics (reviewed by (Ypsilanti et al., 2010)).

1.1.5 Synaptogenesis

When the axon has reached its target, **synapses** are formed with the target cell(s), which must be strengthened and maintained in order to survive. Although advances have been made in understanding the organization and the main functions of the synapse, our understanding of the processes of synapse formation and maintenance – synaptogenesis – is still incomplete (see (Petzoldt and Sigrist, 2014; Südhof, 2018)).

As a first step, the prospective presynaptic and postsynaptic membranes must be identified and matched, and an initial contact must be formed. A range of membrane proteins and pathways are used for this purpose, including the synaptic cell adhesion molecules (a subgroup of the IgG superfamily CAMs), neurexin-neuregulin and Eph-ephrins (Petzoldt and Sigrist, 2014). Interestingly, many of these early synaptogenic proteins play additional roles in axonal branching and pathfinding (Petzoldt and Sigrist, 2014) (see also **figure 2**). This principle of reusing molecules in different stages of development for different processes often occurs in development, as described by (Wolpert and Tickle, 2011):

"The fact that, at any given time, an inductive signal selects one out of several possible responses has several important implications for biological economy. On the one hand, it means that different signals can activate a particular gene at different stages of development: ***the same gene is often turned on and off repeatedly during development****. On the other,* ***the same signal can be used to elicit different responses in different cells****. A particular signaling molecule, for example, can act on several types of cell, evoking a characteristic and different response from each, depending on their developmental history. As we will see in future chapters, evolution has been lazy with respect to this aspect of development, and* ***a small number of intercellular signaling molecules are used again and again for different purposes*** *(bolding by author)."*

The genetic redundancy of molecules used for the initiation of the assembly of synapses is also noteworthy (Petzoldt and Sigrist, 2014; Südhof, 2018).

In the second step, active zone scaffold proteins must accumulate at the putative synaptogenic sites after the establishment of transsynaptic contact and communication (Petzoldt and Sigrist, 2014). Finally, the active zone must mature in a series of reorganization steps from loose to tight coupling of Ca^{2+} channels

and synaptic vesicle Ca^{2+} sensors to enable the incredibly fast and highly controlled synaptic coupling of mature synapses (Petzoldt and Sigrist, 2014).

1.1.6 Synapse elimination

Synaptogenesis in the developing brain "overshoots": redundant synaptic connections are formed, which are only transiently present (Kano and Watanabe, 2019; Purves and Lichtman, 1980). While some synapses are strengthened, redundant synapses are weakened and eventually eliminated (reviewed by (Kano and Hashimoto, 2009)) by a process called **synaptic pruning** (see **figure 5A**). This process has been studied in model synapses in the brain, including the climbing fiber (CF) - to - Purkinje cell (PC) synapse in the cerebellum, and the retinogeniculate synapse between retinal ganglion cells and the thalamic neurons of the dorsal lateral geniculate nucleus (dLGN) (see (Kano and Watanabe, 2019)).

In both the cerebellum and the dLGN neural activity is crucial for synaptic remodeling, which can be spontaneous activity and/or external drives (somatosensory input) (Kano and Watanabe, 2019). Several trans-synaptic molecules, such as semaphorin 3A and 7A, have been found to be involved in synapse strengthening or elimination of the CF-to-PC synapse (Kano and Watanabe, 2019). Intriguingly, both semaphorins are expressed by PCs (albeit at different time points) and signal retrogradely to the CF but with opposing effects on synapse elimination (Uesaka et al., 2014). Anterograde signaling also plays a role, as indicated by the discovery that signaling of C1ql1 from CFs through its postsynaptic receptor on PCs is required to determine and maintain the single remaining ("winner") CF (Kakegawa et al., 2015).

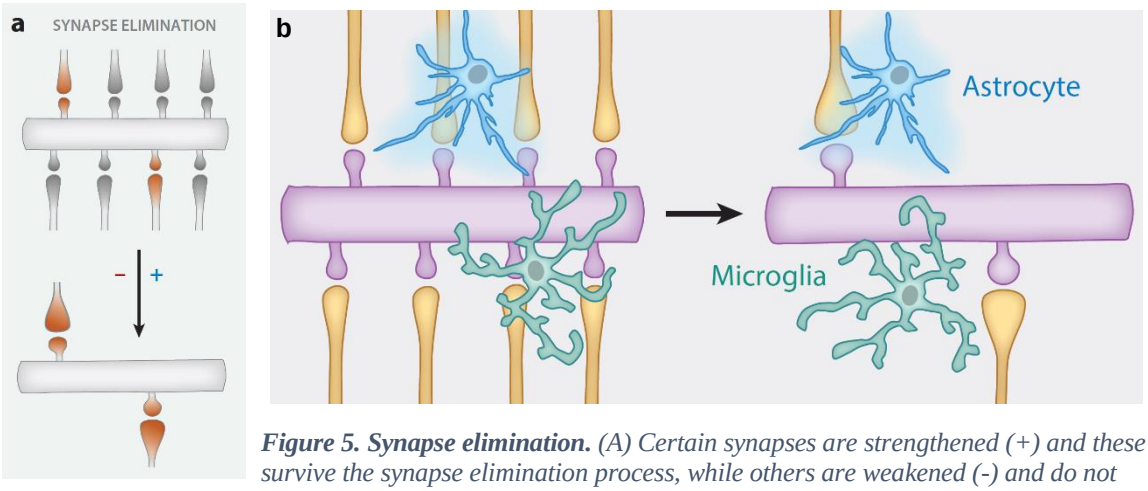


Figure 5. Synapse elimination. *(A) Certain synapses are strengthened (+) and these survive the synapse elimination process, while others are weakened (-) and do not survive. (B) Synapse elimination is (partially) mediated by astrocytes and microglia. Figure adapted from (Wilton et al., 2019).*

Interestingly, many molecules implicated in remodeling the retinogeniculate synapse are until now immune-related, and microglia and astrocytes directly phagocytose unwanted synapses and secrete

molecules controlling synapse phagocytosis (see **figure 5B** and (Kano and Watanabe, 2019)). Glial cell involvement for synaptic pruning has been found in other circuits too (Neniskyte and Gross, 2017), as well as in different organisms (Wilton et al., 2019). Again, parallel, redundant pathways lead to synapse remodeling, partially mediated by molecules that are also involved in other developmental processes.

1.1.7 Programmed cell death

Like synapses, neurons themselves are also produced in excess, followed by regulated elimination by apoptosis, a term coined by Kerr and colleagues (Kerr et al., 1972). A large part of neurons in the PNS (up to 50%) and in some areas of the CNS is eliminated around the time connections are made with other cells (reviewed by (Dekkers et al., 2013; Oppenheim, 1991)). **Programmed cell death** (Lockshin and Williams, 1964) during neuronal development was long thought to stem from the competition for survival factors. Based on an experiment where removing the hindlimb in the chick embryo reduced the number of sensory and motor neurons (Hamburger and Levi-Montalcini, 1949), the "neurotrophic factor hypothesis" postulated that target-derived factors are required for the survival of neurons, and that this signal travels retrogradely through the axons (Harrington and Ginty, 2013; Oppenheim, 1991; Zweifel et al., 2005). Indeed, findings in the PNS support the notion of overproduced neurons competing for limited amounts of nerve growth factor (NGF) produced by target tissues (Ito and Enomoto, 2016; Levi-Montalcini, 1987). In contrast, a recent model suggests that sensory neurons have particular molecular features that predict their probability to respond to environment-derived survival signals (Wang et al., 2019).

Accordingly, single growth factors seem dispensable for survival in the developing CNS, where neuronal activity and other intrinsic mechanisms drive the propensity of neurons to undergo apoptosis (Dekkers et al., 2013). Overall, Yamaguchi and Miura classify the triggers of programmed cell death in nervous system development into several, not always mutually exclusive groups: (1) induction of death ligands and/or pro-apoptotic proteins, (2) loss of survival signals, (3) growth factor signaling, (4) cell-cell interaction and (5) intrinsic transcription factor expression (see (Yamaguchi and Miura, 2015)).

Interestingly, local activation of caspases, the evolutionary conserved family of cysteine proteases mediating apoptosis originally discovered in *Caenorhabditis elegans* (Horvitz, 2003; Nicholson, 1999), seems involved in the local refinement of axons and dendrites in neuronal circuit formation without causing cell death, indicating there is some spatial confinement of apoptotic signals (Dekkers et al., 2013; Yamaguchi and Miura, 2015). Indeed, retrograde signaling during development does not only regulate neuronal survival, but also affects axonal growth and targeting, neuronal specification, and synapse formation (reviewed by (Harrington and Ginty, 2013; Zweifel et al., 2005)).

1.2 THE THALAMOCORTICAL SYSTEM

The processes of axon guidance mechanisms have often been studied in the context of the thalamocortical system, consisting of the aforementioned TCAs as well as corticothalamic axons (CTAs) (see (Lopez-Bendito and Molnar, 2003)). Two broad classes of thalamocortical neurons exist. The first type of specific and topographically organized neurons lies in first order nuclei, including the visual dLGN, the auditory medial geniculate nucleus, the motor ventrolateral nucleus, and finally, the somatosensory ventrobasal nucleus (VB). These neurons mainly project to layer 4 of their respective primary cortical area. The second category of multi-specific and topographically less structured neurons is found in higher order nuclei and project to layer 1 of extensive cortical regions (Grant et al., 2012; López-Bendito, 2018). The TCAs from the thalamocortical neurons in first order nuclei convey information from receptors in the eye, ear, muscle, and skin to the sensorimotor regions of the cortex. The CTAs project from the sixth layer of the cortex back to the first order nucleus in the thalamus from which they received input (Stiles and Jernigan, 2010).

1.2.1 The development of the tactile sensory circuit

The somatosensory circuit, containing the somatosensory VB, has been used in particular as a model system to study the emergence of topographic maps in the cortex and subcortical relays as well as circuit plasticity (reviewed by (Erzurumlu and Gaspar, 2012; López-Bendito, 2018)). During the formation of the mechanosensory brain circuit, the same developmental processes as mentioned earlier take place: neurogenesis, axonal elongation and pathfinding, synaptogenesis, and elimination of redundant or aberrant synapses, all under the influence of familiar molecular cues (Iwasato and Erzurumlu, 2018). As an example, axonal pathfinding was found to be controlled by transcription factors and axon guidance molecules, including Slit and members of the neuroregulin 1 family (López-Bendito, 2018) as well as ephrins-Eph receptors (Erzurumlu and Gaspar, 2012). Here I describe the tactile sensory circuit and its development, as illustrated by **figure 6** (see (Iwasato and Erzurumlu, 2018)):

(1) Neurogenesis of sensory neurons in the trigeminal ganglion (TG) and dorsal root ganglia (DRGs) takes place between E9 and E13.5. Their peripheral sensory axons contain low threshold mechanoreceptors to sense the environment. The central axons of some DRG neurons bifurcate upon reaching the spinal cord, with one part terminating in the dorsal horn and the other ascending to the dorsal column nuclei (DCN). The central axons of TG neurons bifurcate as well, but in the pons, with one descending to the cervical cord and the other ascending to the spinal brainstem nuclei.

(2) There is not much information about the development of the gracile and cuneate nuclei of the DCN which, as said, receive central axons of DRG neurons. The gracile nucleus receives information from the lower body, the cuneate nucleus from the upper body. Neurons in the DCN are estimated to be born between E12-E15. Their axons cross the midline and travel along the medial lemniscus to the ventroposterolateral (VPL) nucleus of the thalamus, but their pathfinding, molecular cues and specific topography along the medial lemniscus have not been studied.

The brainstem trigeminal nuclei which are innervated by the TG neurons have been studied in much greater detail. They consist of the principal sensory nucleus (Pr5) and the spinal trigeminal nucleus (Sp5), and neurogenesis takes place at E10.5-E15.5. The main tactile pathway neurons reside in the Pr5. The ventral part of the Pr5 (PrV2) is derived from rhombomere (r) 2 and receives ophthalmic and maxillary afferents mainly from the whisker pad (Oury et al., 2006) from E12. By P0-P1, a whisker map containing "barrelettes" forms (Ma and Woolsey, 1984), consisting of thalamic-projecting neurons and afferent terminals from the TG. The dorsal part of the Pr5 (PrV3), derived from r3, receives mandibular afferents instead (Oury et al., 2006). Pr5 axons are sent out from E11 and cross the midline and navigate the trigeminal part of the medial lemniscus to the ventroposteromedial (VPM) nucleus of the thalamus.

(3) The VPL receives afferents from the contralateral DCN, which are topographic: the forepaw region has barreloid-like patches corresponding to the palmar pads. The VPL in turn contains thalamic neurons that project towards the somatosensory cortex in a topographic manner. Like in the VPL, neurogenesis in the VPM takes place between E10-E15.5. The VPM receives afferents from the contralateral Pr5 starting at E17. By P1, Pr5 axons form diffuse terminal fields in the VPM. At P2-P3, whisker-specific patterns begin to emerge and by P4 "barreloids" (Van Der Loos, 1976) have formed in the dorsolateral VPM, which receives maxillary afferents. The ventromedial part is innervated by mandibular afferents. Both the VPL and the VPM, together called the ventrobasal (VB) nucleus of the thalamus, send projections to layer IV of the somatosensory cortex, as discussed before. It is not known whether the projections of the VPL and VPM start out simultaneously, or if one follows the other.

The dominant neurotransmitter of the somatosensory system is glutamate, which is released from afferent fiber terminals at all synapses in the ascending pathways, from the dorsal horn, DCN and thalamus to the cortex (Graziano et al., 2008). Out of the three known vesicular glutamate transporters (VGLUT1-3) that shuttle glutamate into synaptic vesicles in axon terminals, VGLUT1 and VGLUT2 are relatively abundantly expressed throughout the central nervous system. They are expressed by specific glutamatergic neuronal populations and usually do not colocalize in the same neurons/synaptic terminals (Graziano et al., 2008).

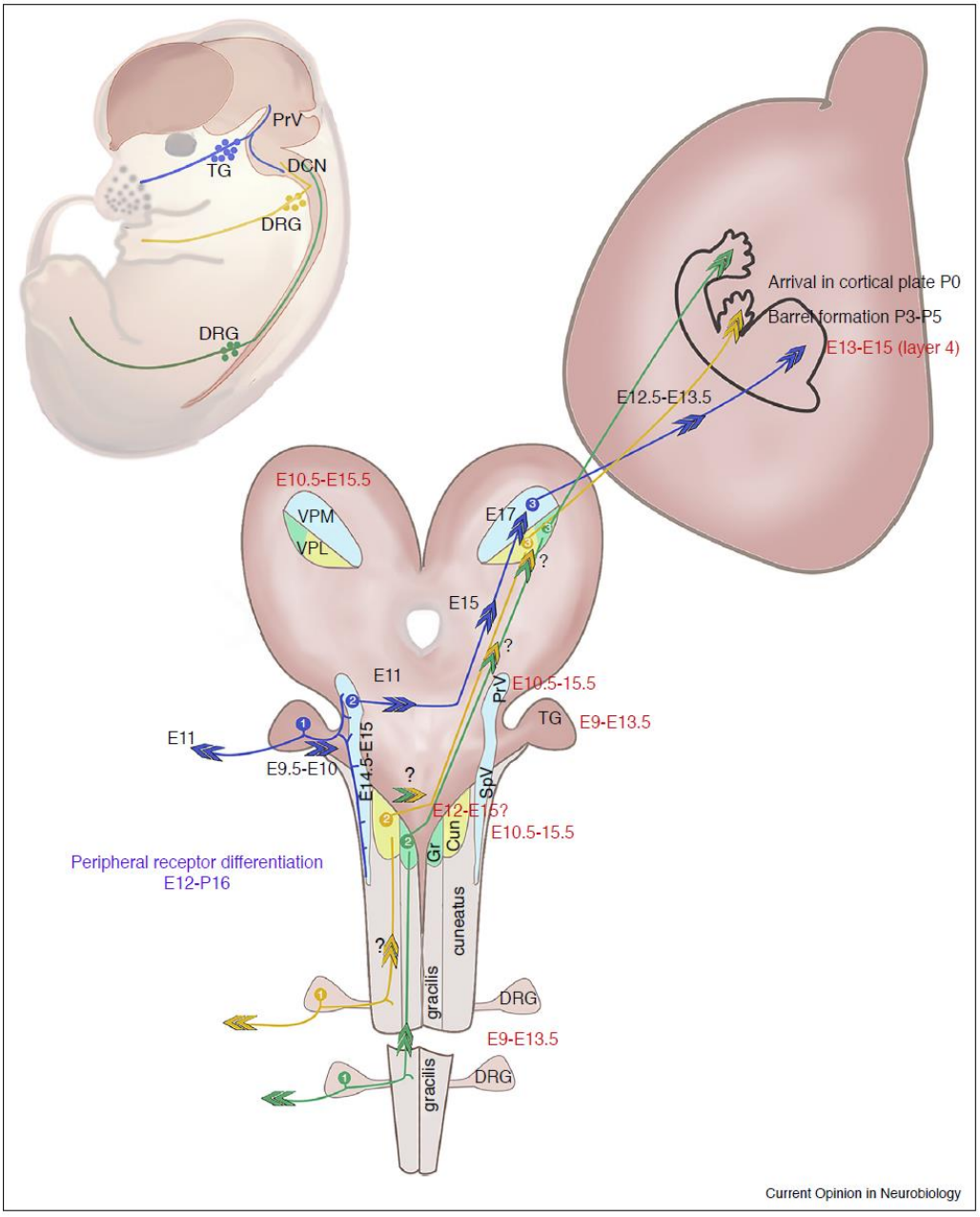


***Figure 6. Development of tactile sensory circuitry**. The cartoon illustrates the timing of neurogenesis (in red), and axon elongation and arrival at target regions (in black). Gr: gracile nucleus, cun: cuneate nucleus. See the text for details. Image reproduced from (Iwasato and Erzurumlu, 2018).*

This is also the case for the somatosensory circuit, where the ascending lemniscal and spinothalamic pathways use VGLUT2, while the descending corticothalamic terminals use VGLUT1 (Graziano et al., 2008). This VGLUT1-expressing corticothalamic innervation of the VB is established between E18.5 and P0.5 (Grant et al., 2012; Jacobs et al., 2007). Inhibitory projections from the thalamic reticular nucleus develop by P7-P8 (Imaizumi et al., 2018), at a time when electrical coupling of VB neurons via gap junctions starts to decrease (Lee et al., 2010).

1.2.2 Synapse elimination in the VPM

In the VPM, synapse elimination takes place after the barreloids have been formed. It was hypothesized that sensory experience before P12-P13 might be required to prime the lemniscal synapse for experience-dependent refinement. This is because an early (from P5 or from P10) or late (from P16) onset of sensory deprivation did not affect the refinement of synapses, while whisker removal started at P12-13 disrupted developmental synapse elimination (Arsenault and Zhang, 2006; Kano and Hashimoto, 2009; Wang and Zhang, 2008). Another possibility is that sensory deprivation started at an early, but not late, age activates mechanisms that compensate for the loss of sensory experience (Wang and Zhang, 2008).

A recent study found that the dorsolateral VPM is innervated by afferents from hindbrain areas other than the PrV2 (i.e., the dorsal Pr5, Sp5 and DCN) right after barreloid formation (Takeuchi et al., 2014). These ectopic afferents are, however, preferentially removed via functional synapse elimination, which was in part mediated by sensory experience (Takeuchi et al., 2014). Interestingly, a similar but long-lasting rewiring takes place after peripheral sensory nerve transection at P21, which might be the neural basis for ectopic pain behavior (Takeuchi et al., 2017; Takeuchi et al., 2012).

Glia play a role in synapse remodeling in the VPM. For example, developing astrocytes produce the cytokine interleukin 33, which promotes engulfment and elimination of synapses by microglia (Vainchtein et al., 2018). Another study found that calcium signaling in astrocytes regulates synapse elimination as well (Yang et al., 2016). In mice lacking the type 2 inositol 1,4,5-trisphosphate receptor, somatic calcium signaling in astrocytes was disturbed, resulting in reduced synaptic remodeling, which could be rescued by infusion of ATP, a known gliotransmitter (Yang et al., 2016).

1.3 ENDOCANNABINOIDS

Endocannabinoids are small signaling lipids that are endogenous ligands of cannabinoid receptors (reviewed by (Kano et al., 2009)). The best described endocannabinoids are N-arachidonoyl ethanolamine (AEA, also known as anandamide) (Devane et al., 1992), and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), although more candidates from their larger families of lipids have been suggested (see (Di Marzo, 2018; Fonseca et al., 2013)). 2-AG and AEA and their "family members" mainly bind to the G-protein coupled receptors (GPCRs) cannabinoid type 1 receptor (CB_1R) and CB_2R (Matsuda et al., 1990; Munro et al., 1993): 2-AG is a full agonist of both CB_1R and CB_2R, whereas AEA is a partial agonist of both receptors. Additionally, they can also bind to other GPCRs (e.g., G protein-coupled receptor 119), ion channels (e.g., transient receptor potential vanilloid type 1) and even nuclear receptors (e.g., peroxisome proliferator-activated receptors) (Di Marzo, 2018).

In the adult brain, the endocannabinoid system modulates the plasticity of many synapses by mediating retrograde synaptic signaling (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). When released by postsynaptic terminals, 2-AG and AEA can bind to presynaptic CB_1Rs. Activation of the CB_1R activates several downstream second messenger signaling pathways (Keimpema et al., 2011), including the phosphorylation of extracellular signal-regulated kinase (Erk) 1/2 (Berghuis et al., 2007; Derkinderen et al., 2003) and the activation of c-Jun N-terminal kinase (Rueda et al., 2000). These signaling pathways of CB_1Rs result in the inhibition of calcium channels and thus reduce inhibitory or excitatory neurotransmitter release probability (Brown et al., 2004). 2-AG is thought to primarily transmit a rapid, transient retrograde signal, while AEA may function as a relatively slow retrograde or even a non-retrograde signal (reviewed by (Ohno-Shosaku and Kano, 2014)).

Whereas CB_1 receptors are expressed both in the CNS and PNS [including by oligodendrocytes (Molina-Holgado et al., 2002) and astrocytes (Metna-Laurent and Marsicano, 2015)], CB_2 receptors are predominantly viewed as the cannabinoid receptors of the immune system, although they are also expressed by some neurons, as well as cultured oligodendrocytes and their precursors (Katona and Freund, 2012; Molina-Holgado et al., 2002; Pertwee, 2008). Even if we here will focus on the effects of the endocannabinoid system on the brain, it is important to note that the same system is associated with peripheral organs as well (see (Maccarrone et al., 2015)).

1.3.1 Production and degradation of endocannabinoids

Endocannabinoids are synthesized on demand from membrane lipid precursors, when and where needed, in response to stimuli (Maccarrone et al., 2015). 2-AG is present at much higher levels (nmol/gram tissue) in the brain than AEA (pmol/gram tissue) (Katona and Freund, 2012). 2-AG is mainly produced by diacylglycerol lipase (DAGL)α or DAGLβ (Bisogno et al., 2003) and mainly degraded by monoacylglycerol lipase (MGL) (Blankman et al., 2007; Dinh et al., 2002), but also by the serine hydrolase α-β-hydrolase domain (ABHD) 6 (Marrs et al., 2010) and ABHD12 (Blankman et al., 2007), as well as fatty acid amide hydrolase (FAAH) (Goparaju et al., 1998).

AEA belongs to the N-acylethanolamines (NAEs), together with amongst others oleoylethanolamide (Rahman et al. 2014). Endogenous levels of NAEs are principally regulated by their shared synthesis and degrading enzymes (Rahman et al. 2014). In most animal tissues, levels of AEA are low compared to other NAEs. AEA synthesis is mainly mediated by N-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD) (Piomelli 2003), although NAPE-PLD-independent enzymatic pathways exist (Leung et al., 2006). The enzyme mainly responsible for the degradation of AEA is FAAH (Cravatt et al., 1996; Fonseca et al., 2013). Overall, these metabolic enzymes regulate the biological availability of

endocannabinoids and are responsible for maintaining the endocannabinoid tone (Maccarrone et al., 2015).

1.3.2 The endocannabinoid system in brain development

The endocannabinoid system, including its receptors, endogenous ligands and synthesis and degrading enzymes, is present during early development and even before neurogenesis (Berrendero et al., 1999; Psychoyos et al., 2012). Interestingly, endocannabinoid signaling differs in the developing versus the adult brain (Keimpema, Mackie, and Harkany 2011). The axonal growth cones of developing neurons become presynaptic terminals in the adult brain. CB_1Rs are found in both growth cones of axons of cortical interneurons and corticofugal axons (Argaw et al., 2011; Berghuis et al., 2007; Keimpema et al., 2010) and presynapses (Kano et al., 2009; Katona et al., 1999). In contrast, DAGLα, the enzyme producing 2-AG, is present in growth cones, but not in presynaptic terminals (Bisogno et al., 2003). This is summarized in **figure 7**.

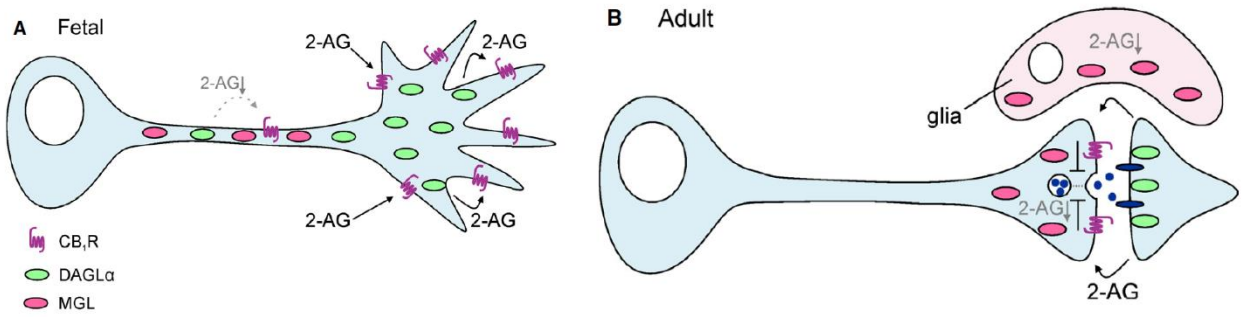


Figure 7. 2-arachidonoylglycerol (2-AG) signaling in the fetal (A) and adult (B) brain. *(A) 2-AG in the growth cone, either coming from other cells (paracrine) or synthesized by diacylglycerol lipase (DAGLa) in the growth cone (autocrine), contributes to growth of the axon and steering decisions by the growth cone. While DAGLa accumulates in the growth cone, monoacylglycerol lipase (MGL) is mainly found in the stabilized axon segment to prevent ectopic signaling of cannabinoid type 1 receptor CB_1R (gray). (B) After the growth cone reaches its postsynaptic target, MGL appears in the pre-synapse, while DAGLa takes place in the post-synapse. In this way, 2-AG can bind to presynaptic CB_1Rs, inhibiting neurotransmitter release (dark blue). MGL is also expressed in glia engulfing the synapse, preventing excess 2-AG to escape the synaptic cleft. Reproduced from (Keimpema et al., 2014).*

In developing neurons, the actions of 2-AG on CB_1Rs are normally spatially tightly controlled by the specific subcellular localization of its synthesis and degrading enzymes, assuring that CB_1Rs are only activated in the developing growth cones to induce neurite outgrowth and affect growth cone steering decisions (Keimpema, Mackie, and Harkany 2011). Δ^9-THC and other exogenous cannabinoids, which are insensitive to degrading enzymes, might however override this spatial control of CB_1R activation, thereby altering positional signaling downstream from CB_1Rs (Keimpema, Mackie, and Harkany 2011).

1.3.3 Endocannabinoid signaling during brain development

Based on pharmacological and genetic manipulation of the endocannabinoid system (see **table 1 and 2**), it was discovered that endocannabinoids are important for the fundamental processes in brain development that were discussed earlier (reviewed by (Maccarrone et al., 2014)). Endocannabinoids influence (1) cell proliferation, migration and differentiation of neurons (Aguado et al., 2005; Berghuis et al., 2005; Berghuis et al., 2007; Mulder et al., 2008; Saez et al., 2014); (2) axonal growth and elongation (Berghuis et al., 2007; Keimpema et al., 2010; Mulder et al., 2008); (3) synaptogenesis and target selection *in vivo* (Berghuis et al., 2005; Keimpema et al., 2013; Mulder et al., 2008); while their effects on apoptosis/survival depend on the cellular context (Guzmán et al., 2001; Wójcik et al., 2020). Additionally, endocannabinoid signaling, via both CB_1R and CB_2R, promotes the survival, proliferation, migration, and differentiation of OPCs in vitro (Gomez et al., 2010; Ilyasov et al., 2018; Molina-Holgado et al., 2002; Sanchez-Rodriguez et al., 2018). This involvement of the endocannabinoid system in multiple phases of brain development is also important for possible consequences of cannabis abuse by pregnant women.

Table 1. The effects of genetic manipulation of the cannabinoid system on the prenatal brain. *CB_1R = cannabinoid type 1 receptor; NPCs = neural progenitor cells; FAAH = fatty acid amide hydrolase; DAGL = diacylglycerol lipase. Based on (Maccarrone et al., 2014).*

KO model	Phenotype	References
CB_1R-KO (global)	Decreased neurogenesis Misrouting of migratory neurons Errors in axonal growth and guidance	(Mulder et al., 2008) (Berghuis et al., 2007) (Mulder et al., 2008)
CB_1R-KO (interneurons)	Misrouting of migratory neurons Change in synaptic distribution	(Berghuis et al., 2007) (Berghuis et al., 2007)
CB_1R-KO (pyramidal cells)	Premature exit from cell cycle Decreased proliferation of NPCs Errors in fasciculation of corticofugal axons	(Díaz-Alonso et al., 2015) (Mulder et al., 2008) (Díaz-Alonso et al., 2012)
FAAH-KO	Increased neurogenesis Increased proliferation of NPCs Increased radial migration in cortex	(Mulder et al., 2008) (Aguado et al., 2005) (Aguado et al., 2005)
DAGLα-KO	Altered synapse distribution	(Keimpema et al., 2013)

Table 2. The effects of prenatal pharmacological manipulation of the cannabinoid system on the prenatal brain. *Δ^9-THC = Δ^9- tetrahydrocannabinol; CBR = cannabinoid receptor; FAAH = fatty acid amide hydrolase. Based on (Maccarrone et al., 2014).*

Prenatal pharmacological treatment	Phenotype	References
Δ^9-THC (agonist CBRs)	Misplacement of interneurons Error in axon fasciculation	(Berghuis et al., 2005) (Tortoriello et al., 2014)
SR141716A (antagonist CB_1R)	Error in axon fasciculation	(Mulder et al., 2008)
AM251 (antagonist CB_1R)	Error in axon fasciculation	(Tortoriello et al., 2014)
URB597 (inhibitor FAAH)	No developmental phenotype	(Wu et al., 2014a)
WIN 55,212 (agonist CBRs)	Disrupted neuronal migration	(Saez et al., 2014)

Focusing on the example of axonal growth, endocannabinoids function as axon guidance cues for interneurons (Berghuis et al. 2007). Additionally, endocannabinoid signaling is important for long-range axon patterning (Mulder et al. 2008) as well as growth cone navigation and axonal elongation of cortical pyramidal cells (Keimpema et al. 2010). Moreover, CB_1R signaling is required for proper axon fasciculation, as pharmacological and genetic inhibition of endocannabinoid signaling results in errors in axon fasciculation (Díaz-Alonso et al., 2012; Mulder et al., 2008; Tortoriello et al., 2014), also in non-mammalian vertebrates (Watson et al., 2008).

The mechanism(s) behind these effects of endocannabinoid signaling on axonal pathfinding and fasciculation is however less clear. As we have seen before, neurons do not develop in a vacuum, but they interact with molecules in the extracellular environment to receive external cues. We also earlier discussed that glial cells are already present in the embryonic brain and that they help with several crucial processes in neuronal development. Therefore, we investigated the molecular interplay of endocannabinoids and glia and its effects on axonal growth in **study I**.

1.4 NEUROPEPTIDES

Neuropeptides are 5-50 amino acid-long linear polypeptide gene products (Hoyer and Bartfai, 2012). They make up a very diverse class of signaling molecules in the central and peripheral nervous system: up till now, approximately 70 neuropeptide genes have been identified in the mammalian brain, divided

into roughly 18 families, see http://www.neuropeptides.nl/tabel%20neuropeptides%20linked.htm (Burbach, 2010). Neuropeptides are expressed across a whole range of species, from *Caenorhabditis elegans* and *Drosophila melanogaster* (Hewes and Taghert, 2001; Nässel and Zandawala, 2019) to humans (Sjöstedt et al., 2020). Some neuropeptides have a highly conserved structure that is shared throughout the bilateria, such as vasopressin and oxytocin, while other neuropeptides share less sequence similarity between different phyla, making genome sequencing necessary to unravel links between them (Semmens and Elphick, 2017).

1.4.1 Neuropeptides versus neurotransmitters

Classical neuropeptides are defined as being 1) synthesized by neurons, 2) released by neurons in a regulated fashion and 3) acting on specific receptors in the nervous system (and on certain peripheral tissues) (Burbach, 2010). From this definition, differences between classical neuropeptides and fast neurotransmitters are not evident, since all these requirements seem true for the latter class as well. Still, the mechanisms of and structural requirements for production, release and effects on target cells are different for neurotransmitters and neuropeptides (see **figure 8**). These differences reflect the unique roles of both classes of signaling molecules in neuronal communication: as Ludwig and Leng wrote, while "neurotransmitters are ´private´ messages passed from one neuron to another, (neuro)peptides are messages between populations of cells" (Ludwig and Leng, 2006).

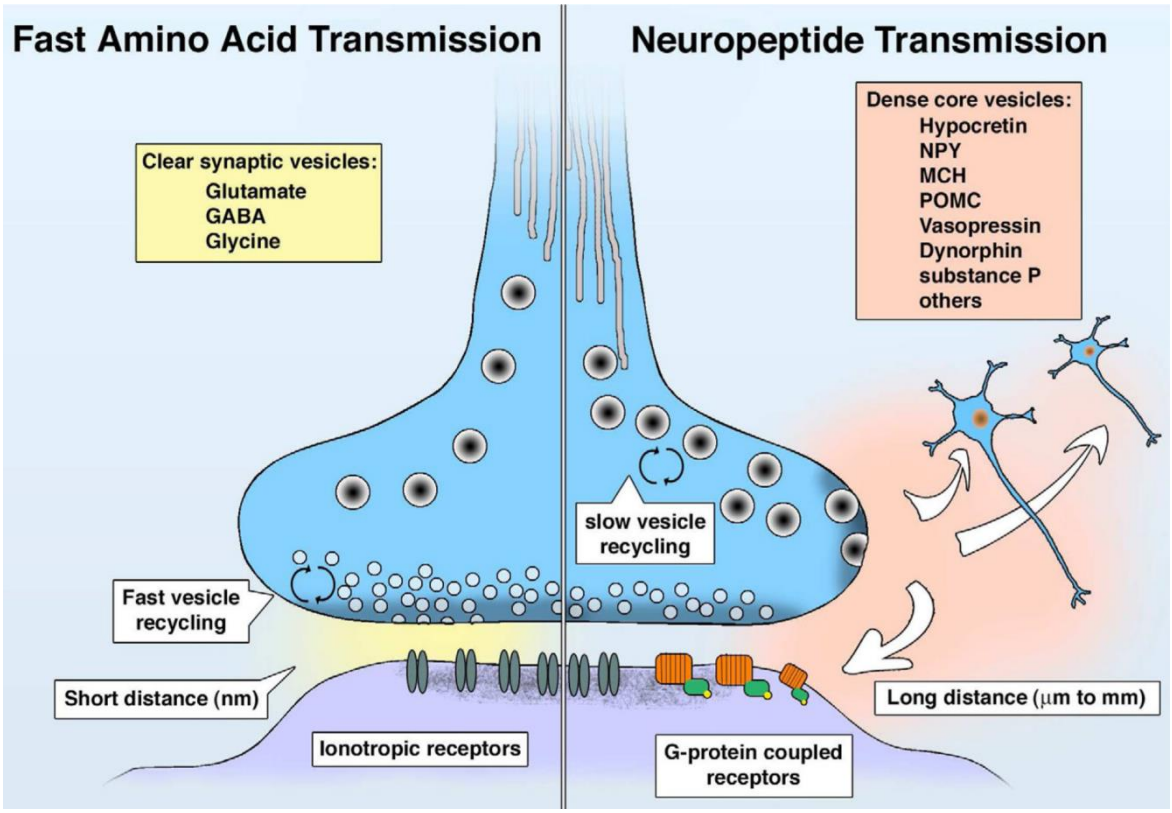


Figure 8. Differences between fast amino acid transmission and neuropeptide transmission. *Image reproduced from (van den Pol, 2012).*

On the one hand, amino acid neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA), and glycine, are produced in axon terminals by enzymes present there and shuttled into synaptic vesicles by transporters, such as VGLUT1-3 for glutamate. Amino acid transmitters are called "fast" because they are subjected to rapid release from synaptic vesicles in the axon terminal and exert immediate effects on postsynaptic neurons. Amino acid transmitters typically bind to postsynaptic ionotropic receptors inducing rapid changes in ion conductance or, less common, to GPCRs, slow-acting metabotropic receptors that activate G protein signaling, whereas monoamines preferentially act via GPCRs. Both types are quickly cleared from the synapse by reuptake transporters or degraded extracellularly (reviewed by (Burbach, 2010; van den Pol, 2012)).

On the other hand, neuropeptides are produced on the rough endoplasmic reticulum from large mRNA precursors encoding prepropeptides, usually 100-250 amino acid residues in length (Hallberg, 2015; Mains et al., 1987; Noda et al., 1982). The prefix "pro" means that the activation of peptides requires cleavage by convertases/endopeptidases (Seidah and Chrétien, 1999), which are already present during neuronal development (Zheng et al., 1994). "Pre" stands for a protein precursor that is secreted from the cell and contains a signal peptide targeting it for release. Gene transcripts of neuropeptides can have splice variants resulting in the expression of different peptides from a single precursor depending on their processing. David de Wied, who coined the terms "neurogenic peptides" (in the 1960s) and "neuropeptides" (in the 1970s), included this in his "neuropeptide concept" (de Wied, 1987):

"*Neuropeptides are synthetized in large precursor proteins, and several are formed in the same precursor such as ACTH and β-endorphin in proopiomelanocortin (POMC). A cascade of processes evolves in peptidergic neurons to express the genetic information into biologically active neuropeptides. These processes control the quantities of neuropeptides synthetized as well as the nature of their biological activity, through size, form, and derivatization of the end product. In this way, sets of neuropeptides with different, opposite, and more selective properties are formed from the same precursor.*"

Prepropeptides are packaged into large dense core vesicles (LDCVs) in the Golgi apparatus. The prepropeptides are being processed by endopeptidases in LDCVs, while they are transported down the axons to the nerve terminals. The mature peptides wait in the LDCVs for their release, which often occurs from non-synaptic sites. This indicates that neuropeptides can act upon other nearby neurons (at a distance of a few microns), not necessarily only on postsynaptic cells (van den Pol, 2012). Neuropeptides might even migrate distances of up to 1 mm via volume transmission (Fuxe et al., 2010). Virtually all neuropeptides bind to GPCRs with high affinity (Ludwig and Leng, 2006; van den Pol, 2012). Neuropeptides are degraded by extracellular proteases anchored to cell membranes (Hallberg, 2015), but have much longer half-lives than classical neurotransmitters (Ludwig and Leng, 2006).

1.4.2 One neuron – one transmitter?

It had long been thought that a neuron could only produce one type of chemical messenger, the so-called "one neuron-one transmitter" hypothesis - a concept questioned however by Burnstock in a thoughtful review (Burnstock, 1976). The first indication for the presence of a neuropeptide and a classical transmitter in the same neuron then came from work on guinea pig sympathetic ganglia, where the neuropeptide somatostatin was found in noradrenergic neurons (Hokfelt et al., 1977). Nowadays, the consensus is that most neurons containing neuropeptides also express a slow or fast classic neurotransmitter, or both; vice versa, most neurons containing a fast neurotransmitter very often also express one or more neuropeptides (or other neuromodulators) (Schone and Burdakov, 2012). Neuropeptides can modulate fast neurotransmission: they diversify the cellular output by engaging GPCRs, which can synergize with or antagonize fast neurotransmitter action.

Importantly, as mentioned before, fast neurotransmitters and neuropeptides are not stored in the same vesicles in the cell. Specifically, transmitters like GABA, glutamate, and the monoamines are stored in synaptic vesicles (diameter around 500 Å), whereas the peptides seem to exclusively be associated with LDCVs (see (Hokfelt, 1991)), although monoamines are present in both (Hökfelt, 2009). Therefore, having both a fast neurotransmitter and a neuropeptide present in the same cell but in different vesicles could expand the dynamic range of neuronal signaling. It would allow small molecule transmitters to be released without colocalized neuropeptides, and neuropeptides to maintain transmission when prolonged stimulation depletes the supply of fast transmitters (Schone and Burdakov, 2012).

For example, orexin/hypocretin neurons contain both the excitatory neuropeptide orexin and the excitatory amino acid transmitter glutamate, which signal parallel, non-redundant features to downstream neurons (Schone et al., 2014). Nevertheless, orexin/hypocretin neurons also (can) contain other neuropeptides and transmitters, including dynorphin, galanin, prolactin and pentraxin (Schone and Burdakov, 2012). Perhaps this indicates that direct release of multiple signaling molecules from a single neuron could cause complex interactions and multiple feedback mechanisms, which have not been studied so far (Schone and Burdakov, 2012). Another possibility is specific recruitment of different messengers to specific synaptic or even non-synaptic sites, so a cell can have a combinatorial signaling code.

Indeed, although the neuropeptides vasopressin and galanin are co-expressed in some magnocellular neurosecretory cells (which also co-express other neurotransmitters and neuromodulators, see (Brown et al., 2020)), the dense core vesicles come in three combinations: either galanin alone, both galanin and vasopressin or vasopressin alone (Landry et al., 2003). Intriguingly, the LDCVs containing galanin alone are preferentially found in dendrites, while the LDCVs with vasopressin alone are present in axon terminals, suggesting preferential targeting (Landry et al., 2003). Galanin seems to have an

autocrine/paracrine inhibitory effect on magnocellular neurosecretory cells via galanin receptor 1 (GalR1) signaling (Brown et al., 2020).

In general, neuropeptides are assumed to be released when neurons are highly active, e.g. under high-frequency stimulation or at bursting activity (Lundberg and Hökfelt, 1983). For example, the release of the excitatory peptide orexin/hypocretin requires higher presynaptic activity than transmission of the excitatory neurotransmitter glutamate by the same orexin/hypocretin neurons (Schone et al., 2014).

1.4.3 How to measure the release of neuropeptides?

It is difficult to study the mechanisms of, and responses to, neuropeptide release (see (van den Pol, 2012)), also because the amounts are small. In fact, neuropeptide receptors are sensitive to nanomolar concentrations of ligand. As a proxy for release of any neurotransmitter, the response to the particular neurotransmitter is usually measured, preferably as a change in voltage or current in the postsynaptic membrane (van den Pol, 2012). However, almost all neuropeptides bind to GPCRs, which influence the cell on a slow timescale, ranging from seconds to minutes. Since peptide release is assumed to require high-frequency discharges, the stimulus-response relationship can be complex, and yet the amount of released neuropeptide may be small (van den Pol, 2012). Therefore, recording the response to neuropeptides by electrophysiology is no small feat.

It is nowadays possible to use biosensors to visualize responses or modify parts of GPCRs into fast signaling systems. For example, the engineering of a chimeric rhodopsin-adenosine A_{2A} receptor resulted in a light-sensitive receptor with specific A_{2A} signaling (Li et al., 2015). Another recent technological advancement is the genetically engineered "neuropeptide release reporters" that were shown to allow imaging of release of neuropeptides at synapses in *Drosophila melanogaster* (Ding et al., 2019).

1.4.4 Somatodendritic release of neuropeptides

Adding another layer of complexity, neuropeptides (as other neurotransmitters, see (Ludwig et al., 2016)) can be released from the somatodendritic complex as well, and the regulation of dendritic release can be independent from axonal release in certain circumstances (Ludwig, 1998). In general, neuropeptide release requires an increase in intracellular Ca^{2+} concentrations, just as is needed for neurotransmitter release (Ludwig and Leng, 2006). Transmitter release from synaptic vesicles containing neurotransmitters involves low-affinity Ca^{2+} sensors. These are only sensitive to the high concentrations close to the site of Ca^{2+} entry via voltage-gated ion channels, opened upon the

depolarization of the membrane by an action potential. While LDCVs are located further away from the entrance point of Ca^{2+}, the machinery for neuropeptide release is coupled to high-affinity Ca^{2+} sensors and can thus be activated by intense activation causing a high rate of Ca^{2+} entry. Interestingly, neuropeptide release from dendrites can also be regulated by intracellular Ca^{2+} release from the endoplasmic reticulum (Ludwig and Leng, 2006).

Neuropeptide release has been often studied in magnocellular neurosecretory neurons (Ludwig and Leng, 2006; van den Pol, 2012). These cells contain a much larger number of peptide-containing LDCVs than peptide-releasing neurons in other brain regions, i.e. not projecting to the pituitary, indicating that they are under different spatial and temporal constraints for release (van den Pol, 2012). Overall, both the precise biophysical requirements for and the mechanisms of the release of neuropeptides have not yet been fully elucidated.

1.4.5 Neuropeptides: more than morphological markers?

Perhaps because of these limitations, neuropeptides have often been reduced to morphological markers of neuronal populations, while their actual contribution to the output of these neurons has received considerably less attention. For example, neurons in the medial preoptic area expressing the neuropeptide galanin were found to control parental behavior (Wu et al., 2014b). Optogenetic stimulation (that is, by light pulses exciting channelrhodopsin 2 at nanosecond precision, (Deisseroth, 2015)) of galanin neurons increased parental behavior, while genetic ablation of neurons expressing galanin reduced parental responses (Wu et al., 2014b). Nevertheless, the neuropeptide galanin was again only used as a marker for neuronal populations activated by certain behaviors: the real significance of galanin signaling itself was not examined (nor was it the purpose of that study).

A more recent paper carefully examined the effect of neuropeptides themselves on behavior. Two neuropeptides (Neuromedin B and Gastrin-releasing peptide, members of the Bombesin-like peptide gene family) were identified as markers for neuronal subpopulations in a key breathing control center (Li et al., 2016). These subpopulations projected to the respiratory rhythm generator which expressed the receptors for these neuropeptides. Introducing either neuropeptide in the latter region *in vitro* or *in vivo* induced sighing (activity). Elimination or inhibition of either receptor reduced basal sighing, whereas inhibition of both abolished it. Ablating the receptor-expressing neurons eliminated basal and hypoxia-induced sighing, but initially left breathing otherwise intact (Li et al., 2016).

These elegant experiments indicate that it is the signaling through neuropeptides themselves that is responsible for the sighing behavior, although the neuropeptides seem redundant in the system, providing parallel pathways. However, in these experiments, the pharmacological and genetic

manipulations are based on “all-or-nothing” responses: either they introduce exogenous neuropeptide in the system, or they remove the (signaling of the) receptors completely. This does neither allow for subtle manipulation on biologically relevant time scales as with optogenetic techniques nor can it reliably correlate behavior to activity patterns as can be done with in vivo electrophysiology or fiber photometry.

In general, neuropeptides act as guardians for the nervous system, they become “active” when the nervous system is challenged, e.g. by stress, injury, drug abuse or neuropsychiatric disorders and upon the ensuing increase of neuronal activity (reviewed by (Hokfelt, 1991)). This indicates that their baseline signaling might be low, but their expression can be induced if circumstances so dictate. Galanin provides one example of a neuropeptide whose expression can be increased/induced by several stimuli.

1.4.6 The isolation of neuropeptides

In the 1970s and 1980s, neuropeptide research was booming, because of the identification of many neuropeptides isolated from several organs (see (Klavdieva, 1996)). While most laboratories studied the biological effects of a tissue fraction before the actual isolation of the active molecule at that time, Viktor Mutt and his student Kazuhiko Tatemoto, both working at the Karolinska Institute, developed a clever method of isolating potentially active molecules. Based on the conserved structure of neuropeptides, which very often contain an amidated C-terminal, they first isolated new peptides from tissue fractions and only then studied their biological effects (Tatemoto and Mutt, 1978). This approach was extremely successful and resulted in the isolation of (amongst several others) neuropeptide Y (1982) and galanin (1983).

1.5 THE NEUROPEPTIDE GALANIN

Galanin, a 29 amino acid peptide (30 in humans), was first isolated from porcine intestine (Tatemoto et al., 1983). It was named after its N-terminal **g**lycine and its C-terminal **alanin**e. The N-terminal end is essential for its biological activity and the first 19 amino acids are over 90% conserved from fish to humans (see (Lang et al., 2015)). Galanin was early on detected in the peripheral and central nervous system (Rokaeus et al., 1984). The expression of galanin at the level of mRNA and protein in the adult brain has been mapped in several species, including human (Gentleman et al., 1989), rat (Melander et al., 1986; Skofitsch and Jacobowitz, 1985) and mouse (Cheung et al., 2001; Perez et al., 2001). There are species differences in galanin expression, especially regarding its co-expression with other neurotransmitters and peptides (Lang et al., 2015). Galanin is expressed throughout the mouse brain (Perez et al., 2001).

1.5.1 Galanin receptors

Galanin can bind to three receptors, GalR1-3. Their mRNA expression patterns have been mapped by *in situ* hybridization in the rat (Burazin et al., 2000; Mennicken et al., 2002; O'Donnell et al., 1999; Waters and Krause, 2000): GalR1 seems to be the most widespread and abundant in the rodent brain, while GalR2 is also expressed at the periphery. GalR3 has in the brain the most limited expression pattern. So far, comprehensive studies on the distribution of GalR1-3 in the mouse brain have not been published, beyond the data provided by the Allen Brain Atlas (Lein et al., 2007). Nevertheless, the distribution of GalR1 mRNA in the mouse seems reminiscent to the rat, albeit with additional expression in the caudate putamen and several midbrain regions (Hohmann et al., 2003; Jungnickel and Gundlach, 2005). Our understanding of the subcellular localization of galanin receptors is partly limited, as current antibodies against galanin receptors appear to lack specificity (Brunner et al., 2019; Hawes and Picciotto, 2004; Hawes JJ, 2005; Lu and Bartfai, 2009). However, the generation of GalR1-mCherry and GalR2-GFP mice knock-in mice is a promising development, although GalR2-GFP expression is at the limits of detection (Kerr et al., 2015).

All three galanin receptors are GPCRs, but they differ in their G protein coupling and subsequent signaling arcs (reviewed by (Lang et al., 2015; Webling et al., 2012)). GalR1, signaling through $G\alpha_{i/o}$ proteins, inhibits adenylate cyclase, leading to opening of G protein-gated inwardly rectifying potassium (GIRK) channels. GalR1 activation can stimulate mitogen-associated protein kinase (MAPK) activity, signaling through a protein kinase C (PKC)-independent mechanism as well. Through $G\alpha_{i/o}$ signaling, GalR2 can also activate MAPK via PKC activation, while it can inhibit adenylate cyclase. Additionally, via signaling through $G\alpha_{q/11}$ proteins, GalR2 triggers phospholipase C activity, releasing Ca^{2+} into the cytoplasm from intracellular stores and opening Ca^{2+}-dependent channels. GalR3 signaling properties are less well studied but include signaling through $G\alpha_{i/o}$ proteins, inhibiting adenylate cyclase, resulting in opening of GIRK channels, like GalR1 (Webling et al., 2012). Although its effects evidently depend on the receptor subtype and subsequent second messenger signaling, galanin is mostly an inhibitory neuropeptide (Xu et al., 2005).

1.5.2 Galanin family of peptides

Galanin is not the only member of the galanin family of peptides. Preprogalanin, the 123/124 amino acid prepropeptide encoding galanin, also gives rise to a second peptide, the galanin message-associated peptide (GMAP), see **figure 9** (Rokaeus and Brownstein, 1986). GMAP distribution appears to overlap with galanin in the brain with some heterologous expression (Hokfelt et al., 1992). A third peptide encoded by a different gene, galanin-like peptide (GALP), was identified through its binding to galanin

receptors (Ohtaki et al., 1999). Its expression is limited to the arcuate nucleus, median eminence, infundibular stalk, and posterior pituitary (Webling et al., 2012). A splice variant of GALP named alarin, with a broader expression pattern but no affinity for galanin receptors, has been discovered (Santic et al., 2006), and more recently, a new ligand for GalR2 and -R3 has been identified, spexin (Kim et al., 2014a).

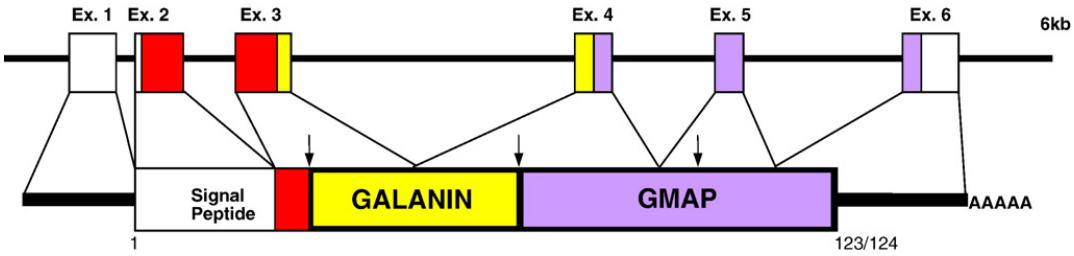


Figure 9. The organization of the preprogalanin gene. *Arrows indicate cleavage sites of endopeptidases. Ex = exon; GMAP = galanin message associated peptide. Image reproduced from (Lang et al., 2007).*

1.5.3 Functional role of galanin

Like other neuropeptides, the expression of galanin can be induced by several stimuli. Studies of galanin knockout (KO) or galanin receptor KO mouse lines and/or galanin-overexpressing mouse lines have provided insights in the involvement of galanin and its receptors in the regulation of many physiological processes (reviewed by (Lang et al., 2015)). These include (but are not limited to) metabolic and osmotic homeostasis, reproduction, pain, arousal/sleep and cognition. Additionally, galanin has been implicated in a range of disorders, from depression to Alzheimer's disease, both in humans and in mouse models of these illnesses (Lang et al., 2015).

Several experimental models show an increase in the expression of galanin upon nerve injury (see (Xu et al., 2010)), although its exact role in nociception is still debated. Galanin axons also hyperinnervate surviving cholinergic neurons in Alzheimer´s disease (Chan-Palay, 1988; Counts et al., 2010). While galanin is depleted in epilepsy (Mazarati et al., 1998), galanin knockout (Gal-KO) mice show increased drug-induced hippocampal cell death in vitro (Elliott-Hunt et al., 2011; Elliott-Hunt et al., 2004) and a higher prevalence of pharmacologically-induced epileptic seizures (Mazarati et al., 2000), likely mediated via GalR2 (Elliott-Hunt et al., 2011; Elliott-Hunt et al., 2004). This implies that galanin has a neuroprotective effect and/or is involved in the neuronal recovery from injury in the peripheral and central nervous system.

1.5.4 Galanin in development

Galanin might have a function in (early) development as well. In cultured embryonic stem cells, galanin is amongst the most abundant ("top three") mRNA transcripts, with GalR1-3 mRNA being expressed as well (Anisimov et al., 2002). Although the cells were cultured in the presence of leukemia inhibitory factor (LIF), known to upregulate galanin expression, galanin expression was also detected in other embryonic stem cell lines under LIF-negative growth conditions (Anisimov et al., 2002; Tarasov et al., 2002). Still, the presence of mature galanin peptide itself was not demonstrated yet, and its presumed functions remained unclear (Tarasov et al., 2002). Interestingly, galanin is indeed upregulated in response to LIF (Corness et al., 1996; Rao et al., 1993; Sun and Zigmond, 1996) as well as estrogens (Kaplan et al., 1988), but downregulated by NGF (Corness et al., 1998; Verge et al., 1995).

Relevant for early brain development, galanin stimulated neuronal differentiation in mouse subventricular zone cultures (Agasse et al., 2013; Cordero-Llana et al., 2014) and in neural progenitor cells isolated from embryonic mouse striatum (Cordero-Llana et al., 2014). Consequently, in Gal-KO mice the rate of adult SVZ neurogenesis falls, resulting in less newly generated cells in the olfactory bulb. Still, Gal-KO mice perform normally in simple olfactory tasks, suggesting redundancy (Cordero-Llana et al., 2014). Galanin also promoted adult neural stem cell survival in a diabetic milieu via GalR3 signaling (Mansouri et al., 2013).

Galanin expression can also be induced in glia and glial progenitors. Galanin and GalR1-2 expression in the subventricular zone, rostral migratory stream and corpus callosum and in OPCs were reported after induction of cortical spreading depression, termed "a benign ´pathophysiological´ stimulus" (Shen et al., 2003). Galanin expression in glial cells had also been observed after intraventricular (icv) colchicine administration (Xu et al., 1992), an effect dramatically attenuated in hypothyroid rats (Calza et al., 1998). These findings were expanded by the notion that OPCs can upregulate galanin downstream of LIF (like in neurons) in response to demyelinating injury, thus increasing survival via autocrine or paracrine signaling (Gresle et al., 2015).

Intriguingly, galanin-like immunoreactivity was reported in tissues of mesenchyme and neural crest origin in mouse embryos from E10-E15, possibly again hinting at a role in stem cell/progenitor proliferation or differentiation (Jones et al., 2009). Similarly, galanin expression was evident in several sensory systems and bone in the rat embryo (Xu et al., 1996). For instance, galanin mRNA and protein are already present at E17 in trigeminal and dorsal root ganglion neurons, and at E15 in the sensory epithelia of the developing ear, eye, and nose, as well as during bone formation at E19 in the rat. GalR1 mRNA was expressed in sensory ganglia of embryos but appeared later in development (Xu et al., 1996). This suggests involvement of galanin in the development of sensory modalities.

Studies using galanin knockout mice have also indicated that galanin is vital for the survival of specific subsets of neurons. For example, galanin knock-out mice are less sensitive to thermal and mechanical

stimuli (Kerr et al., 2000) due to the early postnatal loss of small peptidergic sensory neurons in dorsal root ganglia (Holmes et al., 2000). These effects seem to be mediated by GalR2, as GalR2-KO have the same phenotype (Hobson et al., 2006). Galanin signaling via GalR2 also stimulates neurite outgrowth from adult DRG neurons (Mahoney et al., 2003; Sanford et al., 2008).

The absence of galanin induces the loss of one-third of cholinergic neurons in the basal forebrain, resulting in age-dependent reduced acetylcholine release and loss of spatial memory in 10-month-old mice (O'Meara et al., 2000). Moreover, while pharmaceutical antagonism of GalR2 in neonates reduces the in vivo expression and axonal targeting of vesicular acetylcholine transporter (VAChT), in vitro GalR2 signaling induces extension of the VAChT-containing primary neurite and antagonizes NGF-induced growth cone differentiation (Keimpema et al., 2014), further supporting a role for galanin in axon guidance (Hobson et al., 2013).

Finally, galanin functions as a growth factor for cells producing prolactin, as demonstrated by knock-out mothers who fail to lactate, with their pups dying of starvation or dehydration if not fostered to wild-type mothers (Wynick et al., 1998).

Interestingly, the analysis of the promoter region of the mouse galanin gene indicated that different regulatory elements could control galanin expressed during development versus in the adult after injury (Bacon et al., 2007). This indicates that galanin expression in the injured adult is not just a reactivation of developmental galanin expression (Schwartz, 1992). In other words, neuronal injury does not simply turn back the clock: it is, at least for galanin, a fundamentally different state than “development”.

Importantly, these knockout lines for galanin and its receptors are all constitutive, potentially allowing for developmental compensation: a role of galanin during the development of other brain regions could be masked by the compensatory expression of other, functionally conserved neuropeptides. Nowadays a mouse line containing a conditional knockout allele of *Gal* ($Gal^{tm1a(KOMP)Wtsi}$ from the Knockout mouse project (KOMP) (Skarnes et al., 2011) is available to limit the functional removal of galanin to specific neuronal populations. As an example, this mouse line was recently crossed with a knock-in Cre driver allele under control of the noradrenergic specific *Dbh* promoter (DBH-Cre mouse line) to generate a mouse model in which galanin was selectively disrupted in noradrenergic neurons (Tillage et al., 2020).

1.5.5 Galanin expression in the developing brain

Despite the findings presented above, our understanding of the developmental expression of galanin in the mouse brain is still poor. In the rat, studies on the presence of galanin and its receptors during brain development provided somewhat mixed results, likely due to differences in sensitivity of distinct methodologies. Some researchers only reported postnatal galanin expression (Ryan et al., 1997) or

reported that galanin expression even is 'entirely postnatal' (Sizer et al., 1990), while others found prenatal expression of galanin (Burazin et al., 2000; Gabriel et al., 1989; Wickstrom et al., 2000). Prenatal galanin and GalR1 expression were mostly seen in similar brain regions as in adults (Burazin et al., 2000; Wickstrom et al., 2000). Interestingly, GalR2 expression levels vary during the development of the rat brain, having a broader distribution and a peak in expression before postnatal day 7, particularly in cortex and thalamus, compared to postnatal day 14 (Burazin et al., 2000).

If one of the receptors for galanin is expressed transiently during development, it would make sense that galanin itself is also more broadly expressed in the developing CNS compared to the adult brain; even if there are many examples of peptide-receptor mismatches (see (van den Pol, 2012)). Interestingly, increased levels of galanin peptide were not demonstrated in the thalamus or cortex during the postnatal peak in GalR2 expression in the rat (Burazin et al., 2000). Contrastingly, data from the Allen Brain Atlas from mice seem to agree with transient galanin mRNA expression in several regions, such as the telencephalic vesicle, prosomere 1-3 (p1-3) of the developing diencephalon, midbrain and hindbrain regions, during late embryonic and/or early postnatal development (see https://developingmouse.brain-map.org/gene/show/14195). This transient expression also appears to be reflected in Gal-Cre::tdTomato reporter mice (McCall et al., 2015; Plummer et al., 2015).

These results indicate that the developmental role of the galanin is likely to be complex and to involve multiple tissues and cell types. Although galanin presence has been reported at several stages during brain development, we still do not know what its function is. Therefore, **study II** started with investigating the expression of the neuropeptide galanin in the developing mouse brain.

1.6 HYPOTHALAMUS

Even if there are neuronal populations in all brain areas that express particular neuropeptides, the hypothalamus is particularly diverse, endowed with many small subsets of neurons with unique functional and molecular phenotypes (Ludwig and Leng, 2006). The hypothalamus regulates physiological homeostasis by integrating a range of sensory inputs, comparing them to basic setpoints, and activating autonomic, endocrine, and behavioral responses crucial for survival of the organism and the species (Burbridge et al., 2016). In this way, the hypothalamus controls basic life functions such as energy homeostasis, fluid and electrolyte balance, sleep-wake cycles, stress, thermoregulation, aggression, and reproduction (Saper and Lowell, 2014). It is therefore perhaps not surprising that the anatomy of the hypothalamus is highly conserved across vertebrate species (reviewed by (Xie and Dorsky, 2017)). Like the thalamus, the hypothalamus is organized into a plethora of different nuclei, but unlike the thalamus, these nuclei are heterogeneous and consist of different cell types, whose molecular identity and connectivity have remained largely unknown (Blackshaw et al., 2010).

1.6.1 Molecular dissection of the hypothalamus

The anatomy and the functions of the hypothalamus are complex. While the cortex executes many different functions but consists of a relatively small number of cell types and neurotransmitters that follow a highly stereotyped architecture from one cortical column to the next, the hypothalamus comprises a plethora of cell populations that are distinct in their molecular composition, connections and functions, and are organized in a patchwork manner across the rostro-caudal extension of the hypothalamus (see (Burbridge et al., 2016; Saper and Lowell, 2014)). Indeed, cell migration during development might play a role in this seemingly random organization, perhaps starting from an initial simple, laminar organization stemming from radial migration along the mediolateral axis, which is later overruled by subsequent tangential migration of populations of cells (Burbridge et al., 2016).

While the dissection of the genetic patterning of the developing hypothalamus commenced using microarray analysis and high-throughput *in situ* hybridization (Shimogori et al., 2010), it recently skyrocketed by the use of single cell RNA-sequencing (Romanov et al., 2020). In parallel, our functional knowledge of the myriad hypothalamic circuits has increased by the fact that many of the hypothalamic circuits have distinct neuropeptides as transmitters, allowing for manipulation and mapping of specific populations of neurons using tools such as conditional knock-outs, cells-specific mapping tools and chemo- and optogenetics (reviewed by (Saper and Lowell, 2014)). Indeed, neuropeptides have often been used as mere markers of hypothalamic cell populations, although most functional aspects of neuropeptide signaling have been uncovered in the hypothalamus. An example is the regulation of appetite with roles for amongst others neuropeptide Y (NPY), agouti-related peptide and pro-opiomelanocortin (POMC) (Arora and Anubhuti, 2006).

1.6.2 Endocrine output of the hypothalamus

In addition to contributing to intrahypothalamic pathways and circuits (Hahn et al., 2019; Luiten et al., 1987), different sets of hypothalamic neurons provide autonomic, endocrine or behavioral outputs (Saper and Lowell, 2014). The endocrine output from the hypothalamus comes in three forms. The first is the autonomic innervation of endocrine glands. The second mode is the magnocellular system, in which large neurosecretory cells principally located in the supraoptic and paraventricular nuclei release, primarily, oxytocin or vasopressin directly into the blood vessels of the posterior pituitary gland (although they also secrete these neuropeptides somatodendritically for autocrine/paracrine regulation of the activity of magnocellular neurosecretory cells, see (Brown et al., 2020; Ludwig et al., 2002)). Finally, the third mechanism consists of the neurosecretory parvocellular system, whose smaller neurons in the preoptic area and around the third ventricle send axons to the emergence of the pituitary

stalk, called the median eminence. Here they secrete releasing- or release-inhibiting hormones into the hypophysial portal vessels followed by transport to the anterior pituitary gland, where they control the secretion of pituitary hormones, e.g. gonadotropins, adrenocorticotropic hormone (ACTH), thyroid stimulating hormone, growth hormone and prolactin (Saper and Lowell, 2014).

1.6.3 Dopaminergic populations in the hypothalamus

Contributing to the complexity of hypothalamic outputs, the hypothalamus also contains dopaminergic neurons, which were classically subdivided into five cell groups (Björklund and Dunnett, 2007; Dahlstrom and Fuxe, 1964; Hökfelt et al., 1984). The diversity of their functionality and connectivity is exemplary for the intricacy of the hypothalamus (Romanov et al., 2019):

- A11 cells are located along the periventricular gray matter of the caudal thalamus, medially to the mammillothalamic tract; they project to the spinal cord and use dopamine as their transmitter (Björklund and Skagerberg, 1979; Koblinger et al., 2014).
- A12 neurons lie in the dorsal arcuate nucleus and release dopamine into the portal blood (Björklund et al., 1973), which controls the release of prolactin (reviewed by (Lyons and Broberger, 2014)). They are also called tuberoinfundibular dopamine neurons. Recently, they were also found to increase food intake by stimulating NPY^+ neurons and inhibiting $POMC^+$ neurons via differential dopamine receptor signaling (Zhang and van den Pol, 2016).
- A13 cells lie in the zona incerta, project to the midbrain periaqueductal gray and use dopamine as their transmitter (Björklund and Nobin, 1973).
- The neurons in the periventricular nucleus belong to the A14 cell group and, like A12 neurons, release dopamine into the portal blood (Björklund et al., 1973).
- Finally, A15 is the most rostral group. Dopaminergic neurons in the anteroventral periventricular nucleus stimulate parental behaviors in females, while they inhibit intermale aggression by innervating $oxytocin^+$ magnocellular neurosecretory cells in the paraventricular nucleus (Scott et al., 2015).

Importantly, compared to the original mapping of monoaminergic neurons in the hypothalamus (Dahlstrom and Fuxe, 1964), there are more neurons that express tyrosine hydroxylase (TH), the rate-limiting enzyme for the production of catecholamines, including dopamine, noradrenaline, and adrenaline (see (Björklund and Dunnett, 2007)). Some of these TH^+ neurons, in the hypothalamus mainly located in the latero-ventral aspects of the arcuate nucleus, do not necessarily express any other dopaminergic markers, so the conclusion can be drawn that “the expression of TH is not sufficient in itself to prove that a neuron is catecholaminergic, let alone dopaminergic” (Björklund and Dunnett, 2007). TH has four serine residues that can be phosphorylated by protein kinases in vivo and in vitro

(Dunkley et al., 2004). These serine residues are Ser 8, Ser19, Ser 31 and Ser40, with the latter two having the clearest documented effects on the activity of TH (reviewed by (Dunkley et al., 2004; Dunkley and Dickson, 2019)). The molecular diversity of the hypothalamus, including its dopaminergic neurons, is the subject of **study III**.

1.6.4 Connections to the third ventricle

In addition to neurons, microglia, astrocytes and oligodendrocytes, the hypothalamus contains extra cell types compared to the cortex and the thalamus due to its location around the third ventricle: ependymal cells and tanycytes. The latter cells form the wall of the ventral side of the third ventricle and retain characteristics from radial glia, their developmental origin (see (Rodríguez et al., 2019)). Ependymal cells are multi-ciliated cells that form both the dorsal epithelium around the third ventricle and a niche for neural stem cells ("B cells") in the adult ventricular-subventricular zone (Shah et al., 2018).

The choroid plexus, a complex network of capillaries in the ventricles, produces cerebrospinal fluid (CSF) via the ependymal cells and secretes various growth factors (that help maintain the stem cell pool), micronutrients, hormones, neurotransmitters, neurotrophins and peptide hormones (see (Eichele et al., 2020)). These molecules are either produced by choroid plexus ependymal cells, such as ciliary neurotrophic factor (CNTF) (Manthorpe et al., 1980; Severi et al., 2012), or shuttled by them via specialized transporters after release from neurons (Eichele et al., 2020). Another possible way for neuropeptides and neurotransmitters to enter the CSF is via (the axon terminals of) neurons directly contacting the CSF (Eichele et al., 2020). Indeed, volume transmission of the neuropeptides β-endorphin (Veening et al., 2012) and melanin-concentrating hormone (Noble et al., 2018) via the CSF has been proposed as a mechanism to reach distant brain regions, perhaps in parallel to long-ranging axonal projections. In **study IV**, we investigate volume transmission of CNTF as the basis for a new brain circuit linked to stress.

2 AIMS

The overall aim of this thesis work is to study the development and organization of specific mouse brain regions under the influence of endocannabinoids and neuropeptides.

The specific aims are the following:

• To interrogate the effects of endocannabinoid and Slit/Robo signaling on axonal growth **(study I)**.

• To study the neuropeptide galanin throughout the postnatal development of the somatosensory thalamus **(study II)**.

• To analyze the molecular diversity of the hypothalamus and how hypothalamic neurons functionally integrate in hitherto unknown brain circuits **(study III and IV)**.

3 METHODOLOGICAL CONSIDERATIONS

3.1 METHODOLOGICAL LIMITATIONS OF STUDYING ENDOCANNABINOIDS (STUDY I)

Accepting the risk of stating the obvious: since endogenous cannabinoids are lipid molecules, they are not proteins nor are they translated from mRNA transcripts. Therefore, we cannot directly apply the methods we would normally use to study our molecules of interest, like we do in the other studies (II-IV). This includes immunohistochemistry and Western blotting to show and quantify protein expression, and in situ hybridization, quantitative reverse transcription PCR, and single cell RNA sequencing to assess the expression of mRNA. We also cannot directly downregulate endocannabinoids themselves using an siRNA approach.

Another consequence of endocannabinoids being lipid molecules is that their production is not directly regulated by gene regulatory elements like the promoter sequence and transcription factors as for proteins. Instead, metabolic enzymes, responsible for synthesizing endocannabinoids on demand followed by rapid degradation, regulate the biological availability of endocannabinoids and maintain the endocannabinoid tone (Maccarrone et al., 2015). It is important to mention that the precursors and degradation products of endocannabinoids are also biologically active molecules involved in many biochemical pathways (see (Di Marzo, 2018)).

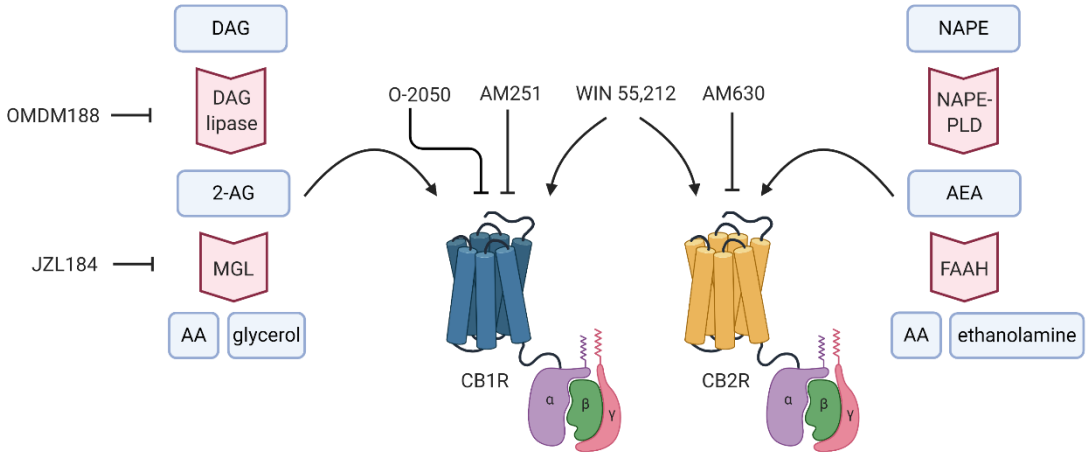


Figure 10. Pharmacological manipulation of CB_1R and CB_2R as used in Study II. *Antagonists of receptors and inhibitors of enzymes are both depicted with arrows ending with a line. 2-AG is a full agonist of both CB_1R and CB_2R, whereas AEA is a partial agonist of both receptors. DAG = diacyl glycerol; 2-AG = 2-arachidonoylglycerol; MGL = monoacylglycerol lipase; AA = arachidonic acid; NAPE = N-acyl-phosphatidylethanolamine; NAPE-PLD = N-acyl-phosphatidylethanolamine phospholipase D; AEA = N-arachidonoyl ethanolamine; FAAH = fatty acid amide hydrolase; CB_1R and CB_2R = cannabinoid type 1 and 2 receptor. Image created with BioRender.com.*

Luckily, we can study the metabolic enzymes and cannabinoid receptors using the molecular biology tools mentioned above as, for example, excellent antibodies are available. Perhaps because the pharmacological effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive component of cannabis (Gaoni and Mechoulam, 1964), led to the search for the cannabinoid receptors before discovering their endogenous ligands (reviewed by (Elphick, 2012; Pertwee, 2005)), there are also many possibilities for pharmacological interventions at the cannabinoid receptors. Some of these drugs were used in **study I** (see **figure 10**).

3.2 CRE MOUSE LINES (STUDY II-IV)

Originally discovered in the bacteriophage P1, the Cre/loxP system allows for the generation of tissue-specific somatic mutations in mice (reviewed by (Kellendonk et al., 1999)). Cre recombinase, a site-specific recombinase, promotes the recombination at specific sequences called loxP, leading to excision of the DNA sequence between two loxP sites (Kellendonk et al., 1999). Therefore, any gene segment flanked by two loxP sites will be deleted in cells expressing Cre recombinase, but will remain untouched in cells that do not express Cre (Kellendonk et al., 1999). Cre has been widely used to introduce specific gene deletions in selected cell populations of genetically modified mice (see (Nagy, 2000)).

Cre recombinase can be inserted into the mouse genome using a knock-in or a bacterial artificial chromosome (BAC) transgenic approach. The latter strategy was pioneered in the lab of Nathaniel Heintz (Gong et al., 2002) and resulted in the Gene Expression Nervous System Atlas (GENSAT) BAC Transgenic Project with the initial goal of developing (a database of) EGFP reporter mouse lines driven by promoters of CNS-specific genes of interest (Gong et al., 2003). The project was soon expanded to also provide the neuroscience community with transgenic mouse lines with CNS-specific promoter-driven Cre recombinase using BAC constructs (Gong et al., 2007). Although the knock-in of Cre seems more likely to result in the recapitulation of expression of the endogenous locus of a gene compared to the relatively random insertion of BAC constructs (Gerfen et al., 2013; Haery et al., 2019), the use of BAC constructs to drive Cre expression allows the targeting of neurons for functional manipulation without affecting the function of the endogenous gene (Gerfen et al., 2013). Advanced recombineering strategies to limit the number of "extra" genes incorporated within the large BAC DNA sequences have been recommended (Ting and Feng, 2014).

In both cases, the Cre mouse lines can be "leaky", i.e. not limited to the endogenous expression of the gene of interest. Therefore, new transgenic Cre mouse lines should be tested before they are used in experiments. The most robust strategy is to cross them to Cre reporter mouse lines and perform (dual) in situ hybridization for the reporter gene and the gene of interest and/or immunohistochemistry for the reporter protein and or the protein of interest (Madisen et al., 2010; Wouterlood et al., 2014). One

"caveat" is that a Cre reporter mouse line cannot distinguish between current and transient, developmental expression of the gene of interest (see **study II**). Transgenic Cre mouse lines can therefore also be characterized by performing Cre-dependent viral transduction in adult mice (Gerfen et al., 2013). Using the same method to trace axonal projections from defined regions and cell types resulted in the Allen Mouse Brain Connectivity Atlas (Oh et al., 2014).

Two mouse lines that I used for the work in this thesis include (1) the GENSAT BAC mouse line galanin-Cre (Ki87, called "Gal-Cre"), which is characterized in **study II**; and (2) the knock-in line DAT-IRES-Cre, where Cre recombinase is driven by the dopamine transporter (DAT) promoter (called "DAT-Cre", **study III**), which has already been carefully characterized in (Papathanou et al., 2019).

Early on, the need for an inducible Cre was recognized (Nagy, 2000), and versions of Cre combined with chemical-inducible systems were developed. One example is the estrogen receptor-tamoxifen system, inducible through the administration of the estrogen antagonist tamoxifen (Indra et al., 1999; Nagy, 2000). However, mouse lines with chemical-inducible Cre did not become as ubiquitous as fast as "ordinary" Cre lines, and they are not available to order from commercial repositories for every gene of interest. This is perhaps due to their inherent technical roadblocks of cytotoxicity, leakiness, off-target recombination, and limited ability to control systems with a high spatiotemporal resolution: problems for which new solutions are still being designed, such as far-red light-induced split-Cre recombinase (Wu et al., 2020).

3.3 VIRAL TRANSFECTION OF THE CNS USING RAAVS (STUDY II-IV)

Recombinant adeno-associated (rAAV) viruses have been used extensively for gene transfer in the CNS, because of the efficient gene transfer, long-term expression of the transgene, minimal integration rate into the genome, low-inflammatory responses, and scalable manufacture (reviewed by (Kantor et al., 2014)). However, rAAV viruses have a limited packaging capacity of 4.7 kb, restricting the use of complex genome regulatory elements (Kantor et al., 2014). Multiple serotypes of AAV exist with specific transduction efficiency and tissue selectivity (Aschauer et al., 2013), because of their differential expression of capsid proteins (Kantor et al., 2014). These capsid proteins determine the interactions with the host cell surface, with most AAVs using cell surface glycans as their receptors for endocytic uptake (Murlidharan et al., 2014).

The serotypes most used nowadays are AAV1, 2, 5, 8 and 9 (see (Haery et al., 2019)). rAAV2 started out as the most widely used AAV serotype to the CNS, but it was later found that other serotypes often have higher transduction efficiencies, a larger spread from the injection site, and higher levels of transgene expression (Aschauer et al., 2013; Burger et al., 2005; Haery et al., 2019). The actual transduction efficiency of the AAV also depends on the anatomical brain region and cell type (Aschauer

et al., 2013), AAV titer and dosage, route of administration (Haery et al., 2019), as well as the mouse strain used (He et al., 2019). Importantly, rAAV viruses can be undergo anterograde or retrograde transport depending on their serotype and can cause toxicity at high doses (Haery et al., 2019).

One original disadvantage of using rAAV viruses to deliver genes into the CNS was that *all* cells in the region of delivery would be transduced, albeit depending on the tropism of the AAV serotype used. Kuhlman and Huang were the first to combine AAV viruses containing a lox site flanking a stop codon to drive expression of fluorescent proteins only in neurons containing Cre recombinase in their genome (Kuhlman and Huang, 2008; Wouterlood et al., 2014). This approach depends heavily on the reliability of the used Cre mouse strain, warranting the need for validation of new transgenic mouse strains (Wouterlood et al., 2014).

Viral strategies can be used to deliver fluorescent proteins for circuit tracing (**study II and III**), but also proteins that allow for manipulation of the transduced cells as in **study IV** (see **figure 11**), such as opsins used for optogenetics, designer receptors exclusively activated by designer drugs (DREADDs) (Alexander et al., 2009; Armbruster et al., 2007) for chemogenetics (reviewed by (Campbell and Marchant, 2018)) and genetically encoded calcium indicators (GCaMPs) (see (Broussard et al., 2014)).

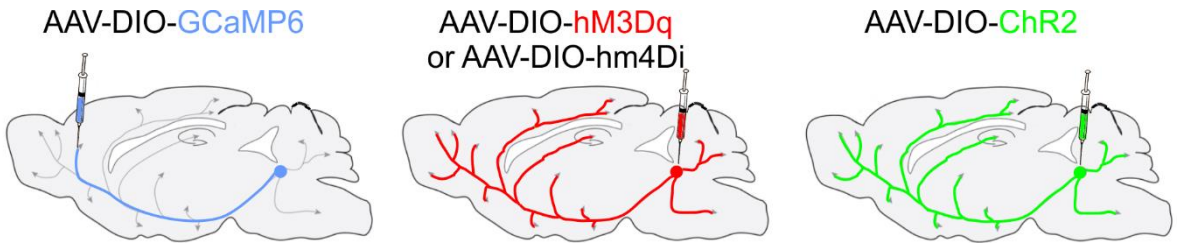


Figure 11. The transduction of long-range projections by rAAV as used in paper IV. *Projections from the locus coeruleus were transduced to express genetically encoded calcium indicators (GCaMP6, left), designer receptors exclusively activated by designer drugs (DREADDs, middle), or opsins for optogenetics (channelrhodopsin, ChR2, right).*

3.4 STEREOTACTIC SURGERIES (STUDY II-IV)

The advantages of using stereotactic surgeries to virally deliver floxed genes (or Cre drivers) to the CNS compared to using recombinant mouse lines are clear: the procedure is both cheaper and faster, the virus can be targeted to specific brain regions at specific times (thereby only manipulating a discrete population of all cells expressing the protein of interest); and it can induce high levels of gene expression (Kuhlman and Huang, 2008). However, there is a transduction gradient from the injection site, stereotactic surgeries are invasive and can damage the region of injection, and some deep brain regions are difficult to reach (Haery et al., 2019). Additionally, variability among viral stocks, injected animals

and number of viral particles present in individual cells can be an issue for functional studies (Madisen et al., 2010).

Another disadvantage is that the standard procedure of stereotactic surgeries is finetuned for the use of adult rodents (Cetin et al., 2006), but the stereotactic frame is not suitable to fix the soft and smaller head of neonatal mice. Earlier protocols to deliver virus particles to the neonatal CNS often rely on intraventricular delivery of the virus in pups at P0-P1 with the aim of widespread transduction of neurons throughout the brain (Kim et al., 2016; Kim et al., 2014b). Another study used the position of blood vessels visible through the skin and skull for the relative orientation of the region of interest, but this is not possible after P3 in pigmented mice such as C57/Bl6 (Davidson et al., 2010). Finally, hypothermia is often used as to anesthetize neonatal mice, but this is only appropriate for mice up to 5 days of age. Since we wanted to virally transduce (pigmented) pups at P7 in **study II**, we designed a 3D printed extension to an existing stereotactic setup which made it 1) suitable to fix the head of neonatal mice (P4 being the youngest age tested) and 2) suitable to be used with isoflurane anesthesia.

3.5 METHODOLOGICAL CHALLENGES OF NEUROPEPTIDE RESEARCH (STUDY II)

The exploration of neuropeptides in the field of *chemical neuroanatomy* has resulted in a wealth of data describing the expression patterns of neuropeptides in the central and peripheral nervous system (and other organs) in several species over the past decades. Still, there have been some methodological challenges, which have hindered progress to study the function of neuropeptides. These include difficulties to (1) visualize neurons expressing neuropeptides, especially their cell bodies; and (2) to visualize neurons expressing neuropeptide receptors, especially their projections, and as mentioned the lack of specific antibodies.

3.5.1 Showing neuronal cell bodies expressing neuropeptides

Firstly, some peptide systems (e.g., oxytocin, vasopressin, orexin) can be readily made visible in the cell body of neurons by immunohistochemistry due to their dramatically high peptide synthesis and actual peptide levels. In contrast, many neuropeptides, including galanin, can in most cases only be shown in neuronal cell bodies by immunohistochemistry after the use of the microtubule inhibitor colchicine (Ljungdahl et al., 1978). Colchicine compromises microtubules and disrupts axonal transport, resulting in the accumulation of neuropeptides in cell bodies; however, it can in fact also alter neuropeptide expression itself (Cortes et al., 1990). Moreover, colchicine interferes with the physiological state of the animal, making it unsuitable to use in studies concerning natural behaviors.

In situ hybridization offers an excellent approach to study the presence of neuropeptides, as demonstrated in the Allen Brain Atlas (Lein et al., 2007), although here only cell bodies are detected. Nevertheless, more physiological methods to display cells (cell bodies) that express neuropeptides are necessary. Luckily, the previously mentioned GENSAT project has produced a large number of BAC-Cre driver lines (Gong et al., 2007), which are now commercially available. By crossing a neuropeptide-Cre driver mouse line with a Cre-dependent reporter mouse line (e.g., *ROSA26Sor*$^{CAG\text{-}tdTomato}$ or similar mouse lines), we can see cells expressing the neuropeptide of interest without having to rely solely on immunohistochemistry (although immunohistochemical enhancement of the fluorescent signal may be necessary). This approach has been used in **study II** for Gal-Cre. In **study III and IV**, Cre driver mouse lines using promoters of other genes of interest (e.g., DAT-Cre in **study III**) were used.

3.5.2 Showing neuropeptide receptors on nerve terminals

The second problem in neuropeptide research is demonstrating the presence of neuropeptide receptors, which are almost exclusively G protein coupled receptors (GPCRs). Although there *are* excellent and specific receptor antibodies available, e.g. for the NK1 (substance P) receptor (Mantyh et al., 1995), and the neuropeptide Y (NPY) subtype 1 receptor (Kopp et al., 2002), antibodies against GPCRs are notoriously difficult to develop and often lack specificity (see (Baker, 2015; Michel et al., 2009)). To detect neuropeptide receptors in tissue, alternative approaches include autoradiography or in situ hybridization techniques. However, the first technique can only distinguish between different receptors for the same neuropeptide, if specific radioligands are available; and the resolution is poor. The latter technique only detects mRNA expression in cell bodies/proximal dendrites, which does not directly correlate with protein expression per se, nor at the cell surface, for example at nerve terminals (Michel et al., 2009).

We used both autoradiography (in collaboration with Robert Ihnatko and Elvar Theodorsson, Linköping University) and an in situ hybridization technique to study galanin receptors in the ventrobasal nucleus of the thalamus (VB) for **study II**, but we were not able to show radioligand binding at any age studied (data not shown). Autoradiography might have a limited sensitivity, seeing that specific [^{125}I]-galanin binding sites could not be detected at all in GalR1-KO mice (Jungnickel and Gundlach, 2005), while GalR2 and -3 have been detected in several brain regions by RT-qPCR (see (Kerr et al., 2015)). For in situ hybridization, we relied on the proprietary probes of the relatively new RNAscope assay. Out of the three galanin receptors, only the probe for GalR1 worked in our and another laboratory (Dereli et al., 2019), again pointing at possible limits of detection.

Another solution to ascertain the expression of neuropeptide receptors would be to use Cre driver lines for neuropeptide receptors as described above or fluorescently tagged neuropeptide receptor mouse

lines. Unfortunately, the commercial development of these mouse lines has lagged. For example, out of the three galanin receptors, only the galanin receptor 3 Cre driver line (GalR3-Cre) is available according to GENSAT (www.gensat.org). The generation of GalR1-mCherry and GalR2-GFP mice knock-in mice is a promising development, although GalR2-GFP expression is at the limits of detection (Kerr et al., 2015). Overall, we understand less of the subcellular localization of neuropeptide receptors than of amino acid receptors (van den Pol, 2012).

3.5.3 Studying the function of neuropeptides

Perhaps because of these limitations, neuropeptides have often been reduced to morphological markers of neuronal populations, while their actual contribution to the output of these neurons has received considerably less attention. In **study II**, we aimed to study the functional relevance of transient galanin expression in the neonatal VB.

Unfortunately, we could not resort to our Gal-Cre driver line combined with viral injections to manipulate our cell population of interest for several reasons. Firstly, to the best of my knowledge, there is no viral Cre-dependent construct available that would hinder only galanin release and not the release of glutamate from VB neurons. Tetanus toxin-light chain (TeLC) for example blocks activity-dependent release from both synaptic and dense core vesicles and thus the release of both neurotransmitters and neuropeptides (Hoogstraaten et al., 2020; Murray et al., 2011; Schoch et al., 2001), even if the SNARE complex proteins for somatodendritic release could differ from axonal release (Brown et al., 2020). Seeing that glutamate release from the TCAs is important for development of the barrel cortex (Iwasato and Erzurumlu, 2018), this could confound the results when using a TeLC approach.

Secondly, since the expression of galanin is transient, we must manipulate galanin (signaling) in neonatal mice, limiting the possibilities for long-term administration of galanin receptor antagonists due to the small size of the mice (e.g., cannula). We could have attempted to administer drugs in organotypic slice cultures, but this would be an ex vivo approach, and we wanted to see what galanin does in vivo.

Because of developmental expression of galanin in other brain regions, we chose to use an in vivo siRNA approach to limit our manipulation of galanin spatially and temporally to only our region of interest. We chose patented Accell SMARTpool siRNA to downregulate galanin, since this product does not require transfection reagents or viral vector transduction, provides a transient downregulation in line with our transient expression and worked well in **study IV** and an earlier study in the laboratory (Romanov et al., 2015). In contrast to **study IV**, we did not inject the siRNA into the ventricles, but directly into the brain parenchyma, as our target, the VB of the thalamus, is not in proximity of any ventricle.

Although we succeeded to inject siRNA against galanin into the VB at P4, we could not confirm convincing galanin downregulation using immunohistochemistry, presumably due to suboptimal dosage and spreading of the siRNA (data not shown). Alternatively, even if we might have succeeded in downregulating preprogalanin mRNA, this might not have resulted in a decreased immunohistochemical signal for galanin in soma, provided that the neuropeptide itself was not released from the neurons. Finally, the downregulation of mRNA might in theory have been overruled by upregulation of galanin due to tissue damage at the injection site. Attempts to confirm the performance of our siRNA per se in the CATH.A mouse cell line (Qi et al., 1997; Suri et al., 1993) failed, because of low endogenous expression levels and incompatibility of the transfection medium and the cell line (data not shown).

4 RESULTS AND DISCUSSION

4.1 STUDY I

As I discussed in the introduction, brain development does not take place in a vacuum: instead, the local environment provides cues for axonal growth and guidance during the formation of brain circuits. Axons finding their way through the extracellular matrix encounter migrating glia and developing oligodendrocytes, which can all be a source of guidance molecules. While a plethora of molecular systems regulating axonal growth has been discovered so far, it is still unclear how these different systems interact (see (Stoeckli, 2018)) or are regulated. We therefore addressed the role of endocannabinoid and Slit/Robo signaling in axonal growth in **study I**.

We enhanced endogenous 2-AG signaling by administering JZL184, an inhibitor of the main degrading enzyme monoacylglycerol lipase (MGL), to pregnant mice of several genotypes (see **table 3**). As expected, only the amount of 2-arachidonoylglycerol (2-AG) increased significantly in the brain of mothers and fetuses, while levels of other endocannabinoids and other hydrolyzing enzymes were not affected. Embryonic exposure to JZL184 resulted in enlarged corticofugal axon fascicles regardless of their projection targets. This effect seemed to be mediated by excessive 2-AG signaling through the cannabinoid type 1 receptor (CB_1R), as untreated CB_1R-KO mice showed the same phenotype. MGL-KO mice did not show errors in axon fasciculation, probably due to CB_1R desensitization. Importantly, JZL184 treatment did not desensitize CB_1R signaling as shown by unchanged levels of degrading and synthesizing enzymes and CB_1R itself in embryonic cortices as well as intact CB_1R signaling in cultured cortical neurons treated with JZL184.

Interestingly, JZL184 administration also induced spreading of axons in the corpus callosum, which coincided with an increased number of oligodendrocyte end-feet, indicating the premature differentiation of oligodendrocytes within the axon bundles. As this phenotype was induced by JZL184 treatment in both wild-type and CB_1R-KO mice, it seemed to be mediated by the CB_2R, which is expressed by oligodendrocytes in addition to CB_1R (Molina-Holgado et al., 2002).

We found CB_1R to colocalize with Robo1 in corticofugal axons in human fetuses and the embryonic mouse brain as well as in cultured cortical neurons. Oligodendrocytes secrete the chemorepellant Slit2, which can bind to the receptor Robo1 on growing axons. Cell culture experiments were therefore used to investigate the connection between endocannabinoid and Slit2/Robo1 signaling.

Table 3. Overview of the effects of prenatal treatment with JZL184 on the brain development of wildtype and several types of KO mouse embryos as reported in study I. *Wt = wildtype; CB_1R = cannabinoid type 1 receptor; 2-AG = 2-arachidonoylglycerol; OPC = oligodendrocyte progenitor cell; CNPase = 2´,3´-cyclic-nucleotide 3´-phosphodiesterase; MBP = myelin basic protein.*

Treatment	Phenotype	Wildtype	CB1R KO	MGL KO	ROBO1 KO
Vehicle (vs. wt)	Overlap CB_1R/Robo1	Yes			
	Overlap CB_1R/Robo2	Yes			
	Fascicle size	Unchanged	Increased	Unchanged	Increased
JZL184 (vs. vehicle)	Increase in 2-AG	Yes			
	Fascicle size	Increased	Unchanged		Unchanged
	Axonal spreading	Increased	Increased		Unchanged
	OPCs: CNPase and MBP	Both increased	CNPase up		CNPase up
	Robo1 in growth cone	Increased			

Firstly, JZL184 treatment of fetal mouse cortices ex vivo induced Robo1 accumulation in growth cone particles. This relocation of Robo1 was shown to depend on CB_1R signaling in cultured cortical neurons, as determined by pharmacology, genetic ablation of CB_1R and siRNA downregulation of MGL. JZL184 treatment also induced Slit2 expression in (the end feet of) oligodendrocytes in a CB_2R-dependent way. Finally, JZL184 treatment mediated growth cone repulsion via Slit2/Robo1 signaling upon neuron-specific downregulation of Robo1 in a co-culture of neurons and oligodendrocytes.

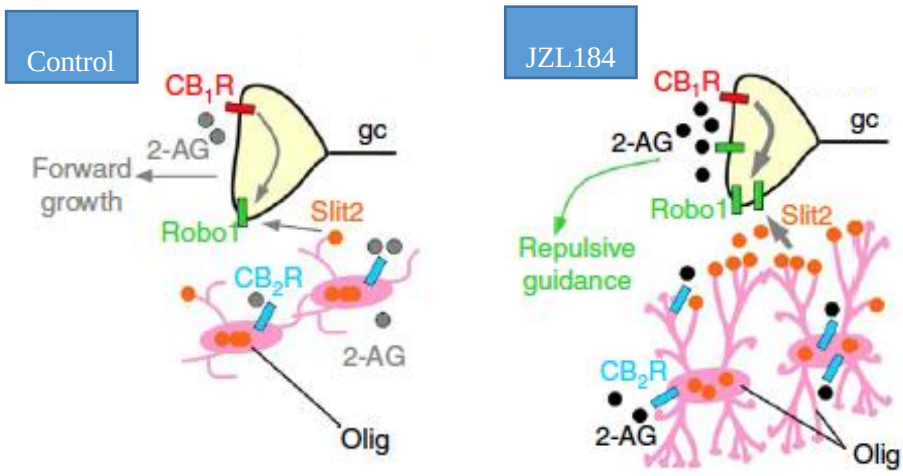


Figure 12. Proposed model of Robo/Slit signaling regulated by CBRs in axonal growth. *For details, see text. Gc = growth cone; Olig = oligodendrocyte; CB_1R = cannabinoid type 1 receptor; 2-AG = 2-arachidonoylglycerol. Figure adapted from (Alpár et al., 2014).*

In this study, I was specifically involved in growth cone particle isolations and Western Blotting. These data contributed to the conclusions that (1) cultured cortical neurons express Robo1 and Robo2 protein; (2) JZL184 administration enriches Robo1 in growth cone particles isolated from fetal cortices; and (3) JZL184 does not change cytoskeletal markers of cultured cortical neurons compared to vehicle treatment.

Endocannabinoids therefore configure focal Slit2/Robo1 signaling to modulate directional axonal growth, by using parallel cell type-specific receptor signaling, according to the model in **figure 12**. These results contribute to our overall understanding of brain development as a multicellular process, with a new way of communication between developing neurons and developing oligodendrocytes. Indeed, this is the first study showing that endocannabinoids can signal through parallel pathways involving multiple cell types and that they can regulate a canonical and well-conserved axon guidance system like Slit/Robo signaling. Although it is unclear what the role of this molecular interplay is under physiological 2-AG concentrations, this study might explain one of the mechanisms of the cognitive deficits seen in children whose mothers used cannabis during pregnancy (Calvigioni et al., 2014; Hurd et al., 2019), especially since its main psychoactive component Δ^9-tetrahydrocannabinol (Δ^9-THC) is resistant to degradation by MGL (Keimpema et al., 2011).

4.2 STUDY II

The neuropeptide galanin is necessary for the development of specific neuronal subsets as indicated by studies on galanin-KO mice. In **study II**, we assessed preprogalanin mRNA levels during brain development. Surprisingly, we found modest preprogalanin mRNA levels in the thalamus at E18.5, high expression in the ventrobasal nucleus of the thalamus (VB) at P7 (which was confirmed by RNAscope, see **figure 13**), and no expression in the adult VB, indicating transient expression in this brain region.

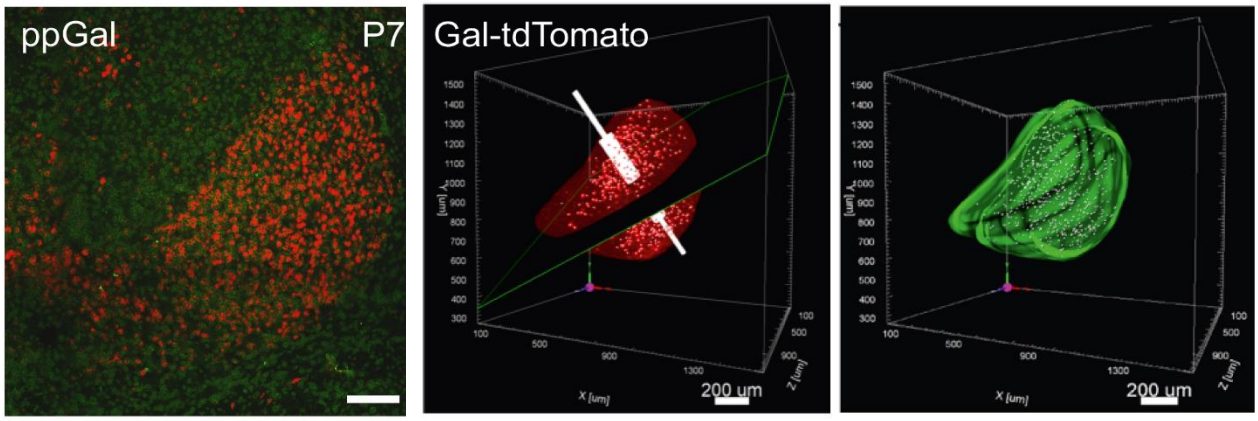


Figure 13. Transient expression of galanin in the somatosensory thalamus. *Preprogalanin (ppGal) expression in the ventrobasal nucleus of the thalamus (VB) at postnatal day (P) 7 as shown by RNAscope (left). iDISCO$^+$ clearing and light-sheet imaging of the VB makes it possible to quantify Gal-TdTomato$^+$ neurons (middle) and represent the 3D structure of the VB (green in right image). Scale bar in the left image is 200 µm.*

We mapped the lifetime expression of galanin in the brain of adult galanin-Cre::tdTomato mice and indeed discovered tdTomato$^+$ cell bodies in the VB, as well as in additional brain regions not known to express galanin during adulthood, which was confirmed by CUBIC clearing and light-sheet fluorescence microscopy. In addition, immunolabeled-enabled three-dimensional imaging of solvent-cleared organs (iDISCO$^+$) clearing of the developing VB detected Gal-tdTomato$^+$ cells (see **figure 13**) already from postnatal day (P) 1. Immunohistochemical analysis of the developing VB showed that galanin peptide was present in cell bodies from P1 and peaked at P4-10 before its expression decreased until P14 (see **figure 14** and **17**). Galanin can also be found in many fine, varicose processes from P10, randomly dispersed throughout the VB.

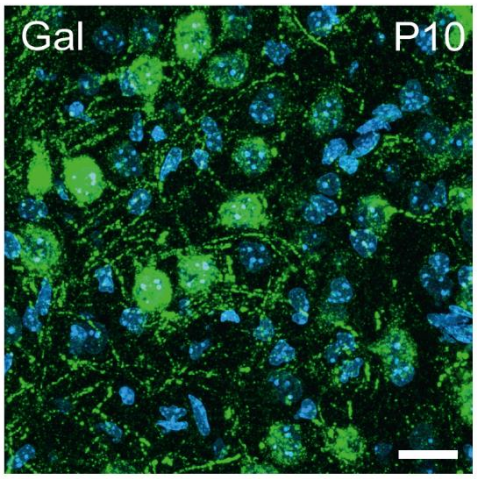


Figure 14. Galanin expression in the somatosensory thalamus as shown by immunohistochemistry. *Galanin (Gal) is present in cell soma and processes at postnatal day (P) 10. Scale bar = 20 µm.*

We designed a 3D-printed extension (see **figure 18**) for the stereotactic apparatus used for microinjections in vivo, so that we could virally transfect cells in young mouse pups at P7, at a time of active expression of Cre. As the VB is also known as the somatosensory thalamus, circuit tracing by AAV-GFP microinjections into the VB of galanin-Cre mice at P7 confirmed that cells transiently expressing galanin in the VB are neurons that project towards the somatosensory cortex, including barrels (see **figure 15**).

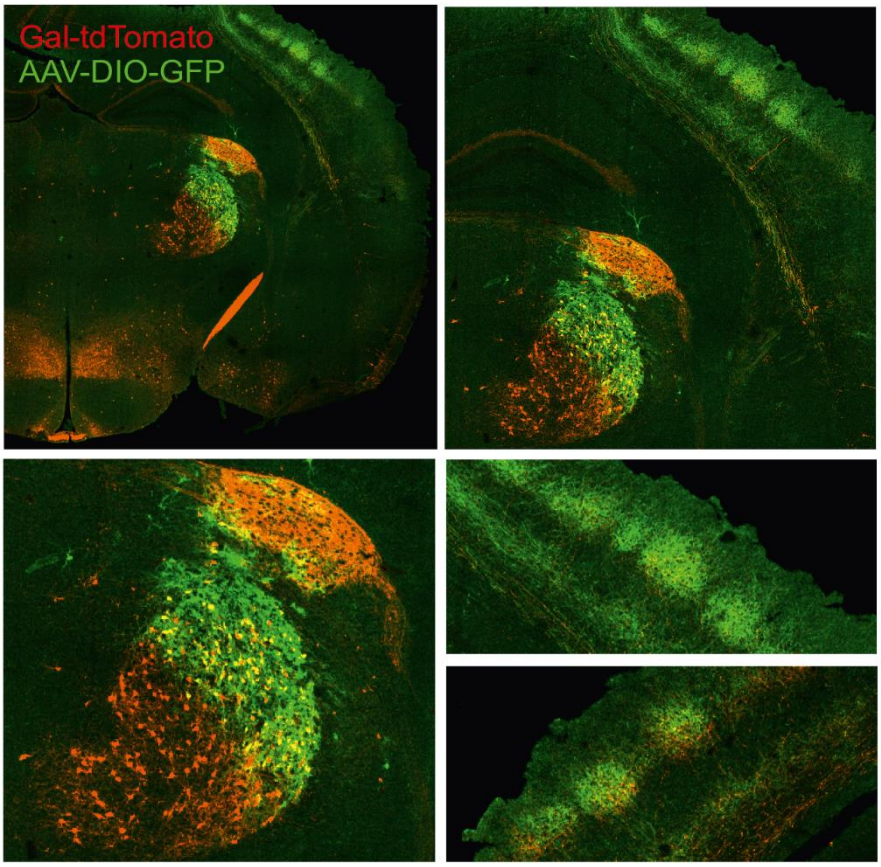


Figure 15. Viral circuit tracing in Gal-tdTomato mice at postnatal day 7. *Viral transduction (green) of Gal-tdTomato$^+$ neurons (red) in the ventrobasal nucleus of the thalamus (VB) shows projections to the somatosensory cortex, including the barrels. Note that Gal-tdTomato expression is also evident in the hypothalamus, optic tract and amygdala.*

Since the actions of a neuropeptide can more depend on the specific distribution of their receptors than on the distribution of their sites of release (Ludwig and Leng, 2006), we next studied galanin receptor expression at P7 and in the adult brain. Using micro-punches, we focused on galanin receptor expression in the VB itself, in one of its downstream hindbrain regions (principal sensory trigeminal nucleus, Pr5) and the projection region (somatosensory cortex) by RT-qPCR (see **figure 16**). Interestingly, we discovered that galanin receptor 1 (GalR1) and GalR3 are expressed at a higher level at P7 in the VB than in the same brain region of the adult, which we could confirm by using RNAscope against GalR1. This finding hints at the potential somatodendritic release of galanin in the VB, with an imaginable effect on the development of VB neurons.

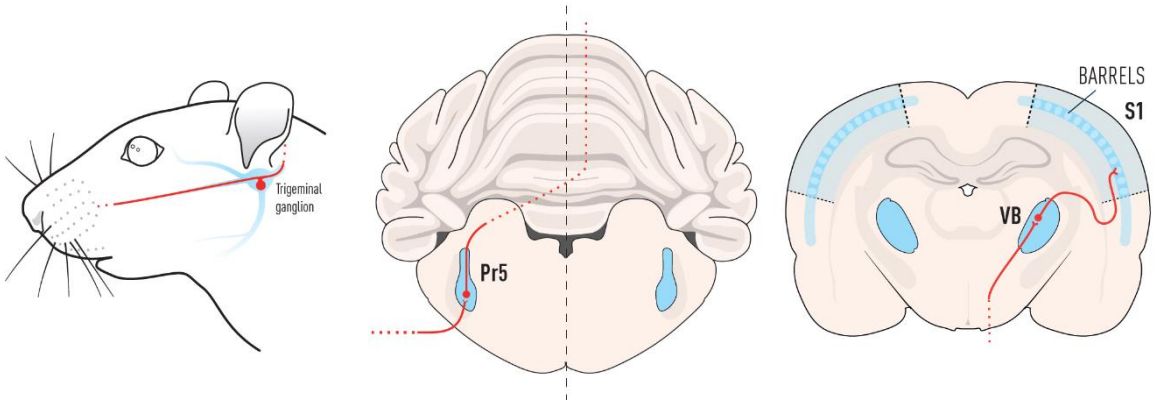


Figure 16. Schematic representation of the tactile sensory circuit, part of which was probed for galanin receptors. *Pr5 = principal sensory trigeminal nucleus; VB = ventrobasal nucleus of the thalamus; S1 = primary somatosensory cortex. Drawing by Matias Karlén.*

During the first postnatal weeks, the afferent projections of the VB are extensively reorganized. We confirmed that the number of vesicular glutamate transporter (VGLUT) 2-expressing puncta decreased drastically from P7 to P10, caused by the loss of small puncta (i.e. <4 um^3). Seeing that galanin is expressed at that time, it might play a role in the selective strengthening and elimination of synapses in the VB (see **figure 17**).

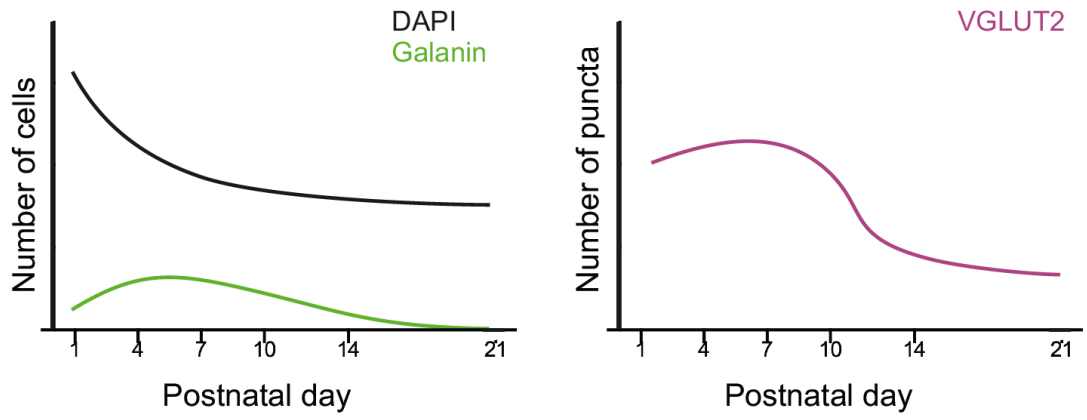


Figure 17. Schematic representation of the change in cell number and number of vesicular glutamate transporter (VGLUT) 2-expressing puncta in the neonatal mouse brain.

This study was the main project of my PhD. I was responsible for the planning, analysis and conceptualization of all experiments presented here (and more which were not included in the manuscript), and performed all experiments, except the design of the 3D extension for the stereotactic instrument, viral transduction in this study, and light-sheet microscopy.

In conclusion, we are the first to show that galanin is transiently expressed in the mouse VB. This transient expression of galanin could either be functional and play a role in development or be "noise" brought about as an inevitable byproduct of a developmental program. Given the energetic costs of producing neuropeptides, we hypothesize that the transient galanin expression is functional, although we have not figured out yet which role it may play. However, taken together with several studies in the literature on other galanin systems described above, the present results support a role of galanin in developmental processes in different parts of the nervous system.

Besides synapse elimination of VGLUT2^{+} inputs, the VB goes through extensive changes throughout the first and second postnatal week, coinciding with the onset of whisking and exploratory behaviors of the pups, highlighting the possibility that galanin is involved in another developmental process than synapse elimination. It would be helpful to assess, if galanin is released from VB neurons and, if so, when and where. Based on the high expression in soma (which is visible without colchicine treatment), we hypothesize that its release might be from soma and dendrites (cf. (Ludwig and Leng, 2006; Vila-Porcile et al., 2009)). Electrophysiological assessment combined with pharmacological manipulations of galanin receptors could help to find out, if galanin can signal through autocrine or paracrine receptors, or instead through receptors on incoming axons from hindbrain regions.

In general, this study showed that studying the lifetime expression of any protein by crossing a Cre driver and a reporter mouse line might bring forth new questions about the possible involvement of that protein in neuronal development, even if transient expression is usually seen as "noise". It remains remarkable how good the brain is at recycling its molecules throughout its lifetime. Perhaps we could learn something from it.

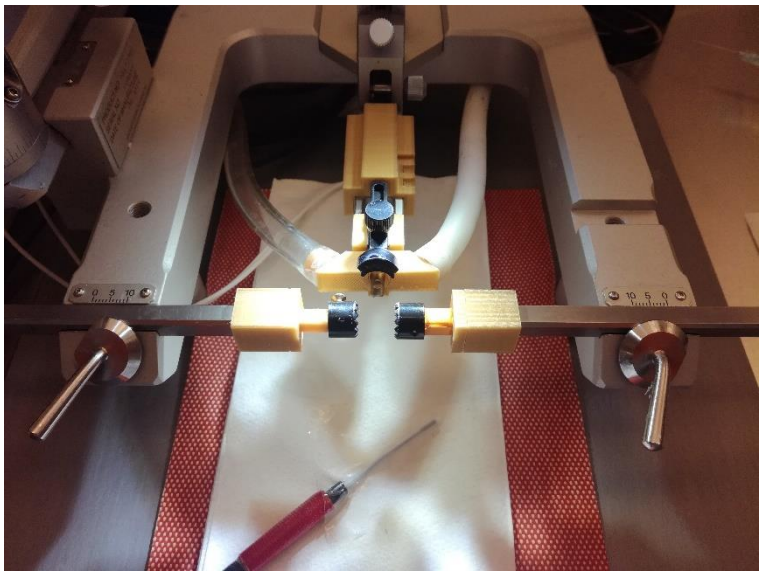


Figure 18. 3D-printed stereotaxic adaptor for precise microinjections in the neonatal mouse brain. *Original design by S. Steffens (2018).*

4.3 STUDY III

The hypothalamus contains the highest diversity of neurons in the brain. Many of these neurons can co-release neurotransmitters and neuropeptides in a use-dependent manner. Until now, the hypothalamus has mostly been studied with the help of candidate protein-based tools to relate behavioral, endocrine and gender traits to specific hypothalamic neurons.

In **study III**, we instead used unbiased single-cell RNA sequencing to map neuronal subtypes in the central column of the hypothalamus. 3,131 single cells were first clustered using the BackSpinV2 algorithm, which sorts cells into nearest neighbors, resulting in seven main cell types. Second-level clustering of hypothalamic neurons (898) classified these cells into 62 clusters, prominently featuring neuropeptides and enzymes involved in the turnover of neurotransmitters (e.g., glutamatergic, dopaminergic or GABAergic markers). Interestingly, some cells were found to exhibit the ability of dual neurotransmission, which was confirmed by Drop-seq and immunohistochemical analysis. Specifically, triple immunohistochemistry for GAD67, vesicular glutamate transporter (VGLUT) 2 and vasopressin in nerve endings at the median eminence revealed that less than 5% of VGLUT2$^+$ terminals also contained glutamate decarboxylase (GAD67), while around 3% of GAD67$^+$ nerve endings also contained VGLUT2. Vasopressin also occasionally coexisted with GAD67 or VGLUT2, indicating that even low mRNA expression can be translated into functional protein and be biologically meaningful.

Additionally, a novel dopaminergic subtype was predicted based on its unique expression of the dopaminergic marker (dopaminergic transporter, DAT) and the transcription factor Onecut3. Using a combination of mouse genetics, immunohistochemistry and brain clearing, these cells were localized to the periventricular nucleus (PeVN), also in human tissue samples. The quest for the circuit integration of the special dopaminergic cells was greatly aided by the advent of a new technique in the lab, stereotactic microsurgeries for AAV transduction. Indeed, viral AAV-GFP transduction in DAT-Cre mice could show that these dopaminergic neurons project ventrally, e.g. to the median eminence, while histochemical detection of TH using the clearing method CLARITY identified potential extrahypothalamic projection sites.

The dopaminergic subcluster uniquely expressed Nmur2, the receptor for the neuropeptide neuromedin S, which is in turn produced by circadian neurons in the suprachiasmatic nucleus (Mori et al., 2005), the circadian clock of the brain. Neuromedin S$^+$ afferents might release the neuropeptide in the CSF for volume transmission, but they also directly innervate DAT$^+$/Onecut3$^+$ neurons in the PeVN. Seeing that the tyrosine hydroxylase (TH) phosphorylation in these neuroendocrine dopaminergic neurons was regulated in a circadian fashion, they might contribute to dopaminergic inhibition of prolactin secretion according to a diurnal schedule. An overview of the proposed new brain circuit is shown in **figure 19**.

In this study, I performed the immunohistochemical analysis, imaging, and quantification of dual neurotransmitters in nerve terminals in the median eminence, as well as the viral circuit tracing in DAT-

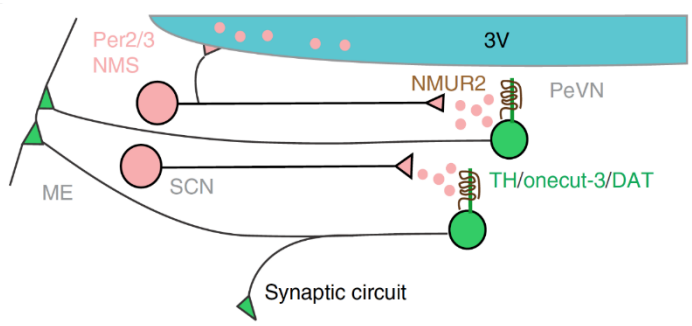


Figure 19. Proposed model of the brain circuit of the potential new dopaminergic cell type described in study III. *Neurons in the suprachiasmatic nucleus (SCN, pink) release the neuropeptide neuromedin S (NMS) onto NMUR2- expressing dopaminergic neurons in the periventricular nucleus (PeVN, green), which in turn project to the median eminence (ME). For details, see text. TH = tyrosine hydroxylase; DAT = dopamine transporter. Figure from* (*Romanov* et al., 2017).

Cre mice. This contributed to the conclusion that (1) hypothalamic neurons are highly flexible in their signaling: they can either co-release neurotransmitters, sort them into subsets of synapses or perhaps switch between modes of neurotransmission depending on their input; and (2) our dopaminergic subtype seems neuroendocrine by releasing dopamine into the portal blood stream to control prolactin release.

Overall, we created for the first time a catalog of neuronal subclasses of the hypothalamus. This provided the field not only with new understanding of hypothalamic organization and function, but also with an opportunity for the prediction-based analysis and validation of (novel) circuits. Indeed, we did not confirm the protein expression of all mRNA transcripts, nor did we prove that the division into the 62 neuronal subclasses that we presented is factual, but we have created a framework for more targeted research to test these assumptions (Romanov et al., 2019). This was illustrated by our proposed circuit linking dopaminergic neurons in the PeVN to the suprachiasmatic nucleus via neuropeptide transmission and (potentially) to prolactin release from the pituitary. The relevance of this data source is further depicted by **study IV**, for which the original hypothesis emerged from the wealth of data accumulated in **study III**.

4.4 STUDY IV

Exposure to stress classically activates the hypothalamus-pituitary-adrenal (HPA) axis to ensure a systemic, acute reaction governed by corticosteroids. In **study IV**, we investigated a potential neural link between the hypothalamus and the prefrontal cortex via the locus coeruleus (LC) for long-lasting stress-induced alertness.

The hypothalamic part of the HPA axis is formed by parvocellular neurons expressing corticotropin-releasing hormone (CRH), which in the classical view release CRH into the hypophysial portal vessels to trigger cells in the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn stimulates the release of corticosterone from the adrenal gland. In this study, CRH^{+} neurons were

found to also project to ependymal cells bordering the third ventricle by using viral RFP-transduction in CRH-Cre mice.

As in **study III**, we used single-cell RNA-seq data to assess the receptor expression of ependymal cells, which were found to mainly express glutamate receptors suggesting a potential response to glutamate released from CRH neurons. Ependymal cells indeed received $VGLUT2^{+}$ nerve endings, responded to the glutamate mimic AMPA ex vivo and, finally, were excited by viral DREADD activation of CRH-Cre nerve endings. Using a combination of mouse genetics and immunohistochemistry, stress was shown to induce both ependymal cell activation as well as the release of ciliary neurotrophic factor (CNTF), a neurotrophin implicated in neurogenesis and repair (Sendtner et al., 1994), into the cerebrospinal fluid. Intracerebroventricular (icv) infusion of CNTF accordingly mimicked stress-induced behavior by inducing hypolocomotion.

Since the LC lies close to the fourth ventricle, is known to be activated by stress, and expresses CNTF receptors (CNTFRs), we hypothesized it to be the next hub in the circuit. Strengthening this idea, icv infusion of CNTF siRNA blocked stress-induced TH and Erk1 phosphorylation in LC norepinephrine neurons. Mechanistically, CNTF links to TH phosphorylation through the Ca^{2+} sensor protein secretagogin (Scgn), seeing that the ablation of Scgn but not CNTF precluded TH phosphorylation. Scgn downregulation in vitro also prevented Erk1 phosphorylation. Moreover, stress-induced TH phosphorylation did not occur in Scgn-KO mice, which were also behaviorally resilient to acute stress. To validate our findings in a physiological context, we combined chemogenetics with behavioral tests (i.e. open field). While chemogenetic activation of LC neurons induced hypolocomotion, inactivation of LC neurons left mice mobile.

Connecting the LC to the prefrontal cortex (PFC), the final link in our hypothesis, we injected biotinylated dextran amine in the PFC and found that it retrogradely labelled $CNTF^{+}$ LC neurons. LC neurons projecting to the PFC also responded to CNTF influx ex vivo as measured by activation of the virally delivered calcium indicator GCaMP. The PFC received TH^{+} efferents containing both phosphorylated TH and Scgn. Scgn-KO mice did not display TH phosphorylation in the PFC in response to stress, nor did they produce the expected local surge in noradrenaline. PFC pyramidal neurons from Scgn-KO mice were also increasingly excitable and more sensitive to noradrenaline. Finally, only long-term activation of efferents of noradrenaline LC neurons in Scgn-Cre mice via channelrhodopsin excited pyramidal neurons in the PFC.

In this study, my contribution was to set up the method of stereotactic surgeries for viral transduction in mice in our lab and teach it to my colleagues. While I did not perform the surgeries for this paper myself, this transfer of knowledge was crucial to be able to transduce and manipulate the specific cells in the different hubs of the new pathway that we propose here. As shown in **study III**, viral transductions can be used to show and/or manipulate the local efferents of a cell population of interest (e.g., in figure 1B, 1F of study IV). Additionally, viral transduction is instrumental to show long-range connections between brain regions, either through (1) injecting the virus at the nerve terminals in the proposed projection area to manipulate the soma of the cells projecting there (e.g., in figure 3G of study IV); or (2) injecting the virus at the soma of the cell population of interest to manipulate the nerve endings in the proposed projection area (e.g., in figure 4H, 6G of study IV).

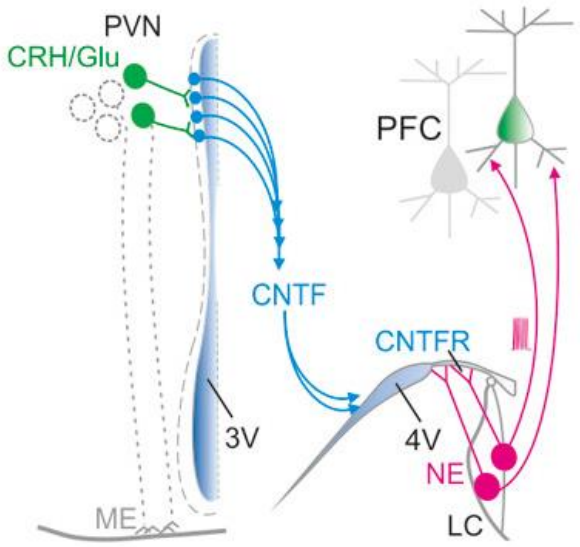


Figure 20. Proposed model of the new stress-related brain circuit described in study IV. *The model connects corticotropic hormone (CRH)-neurons in the paraventricular nucleus (PVN) via volume transmission of ciliary neurotrophic factor (CNTF) in the cerebrospinal fluid to noradrenergic (NE) neurons in the locus coeruleus (LC), which project to the prefrontal cortex (PFC). Glu = glutamate; ME = median eminence; 3V = third ventricle; 4V = fourth ventricle. Figure from (Alpár et al., 2018).*

All in all, this work uncovers a multimodal stress pathway (see **figure 20**) that combines both volume transmission of a neurotrophin as well as synaptic neurotransmission of classical neurotransmitters and monoamines to modulate the excitatory output commands from the PFC. Although this was not the first study to propose volume transmission through the CSF, it is the first linking it to the stress response. Importantly, Scgn is highly expressed in CRH^{+} neurons, and in its absence CRH release at the median eminence and thus the activation of the HPA axis is impaired (Romanov et al., 2015). Therefore, it would be good to untangle the effect of Scgn on the stress response in adrenalectomized mice, as suggested earlier (Pozzi and Matteoli, 2018). Like **study III**, this work showcases the power of "forward transcriptomics" and expands the many flavors of neuronal communication and networks. Seeing the increased prevalence of stress-induced disorders in our society, this new circuit might provide opportunities for drug treatment.

5 CONCLUSIONS

Study I

- The inhibition of monoacylglycerol lipase (MGL) brings about enhanced 2-arachidonoylglycerol (2-AG) levels, resulting in errors in axonal fasciculation and accelerated differentiation of oligodendrocytes within axonal bundles.
- MGL inhibition results in excess production of Slit2 in oligodendrocytes via cannabinoid type 2 receptor (CB_2R) and the repositioning of Robo1 on CB_1R-positive growth cones.
- MGL inhibition induces growth cone repulsion via Slit2/Robo1 interactions.

Study II

- The neuropeptide galanin is transiently expressed in the somatosensory thalamus during postnatal development.
- The functional role of transient galanin expression requires further study.

Study III and IV

- The hypothalamus is molecularly remarkably diverse but can be segregated by looking at enzymes involved in the turnover of neurotransmitters and neuropeptides.
- Single-cell sequencing can help predict brain circuits based on ligands and receptors.
- Hypothalamic neurons functionally integrate in hitherto unknown brain circuits:
    - using neuropeptide signaling (study III)
    - in a complex multi-hub sytem using long-range signaling via volume transmission of a neurotrophin via cerebrospinal fluid (study IV)

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7 POPULAR SCIENCE SUMMARY

The brain is responsible for coordinating the many different behaviors that an individual can perform. Communication between neurons, the nerve cells of the brain, is crucial for this coordination. Neurons can talk to each other in many ways by using different chemical signals. A molecule released from one neuron can bind to its specific "receptor" on the receiving cell, in the same way that a letter can only be delivered to your mailbox if it has your correct address on it. Neurons receive many such signals at the same time and adapt their message to other neurons in return. In this thesis, we have investigated signaling molecules called *endocannabinoids* and *neuropeptides* in four different studies.

Neurons do not only need to communicate to coordinate behaviors in a grown-up individual, but also during the development of the brain. Developing neurons start growing an extension called an axon, which will contact the next neurons (see **figure 1**). The growing axon needs to figure out where to go on the way ("pathfinding") and which other neurons to connect to. For this purpose, chemical signaling molecules are used as well, which either attract the growing axon (like how you are lured to the kitchen by the smell of coffee in the morning) or push it away (like how you avoid a bad-smelling place).

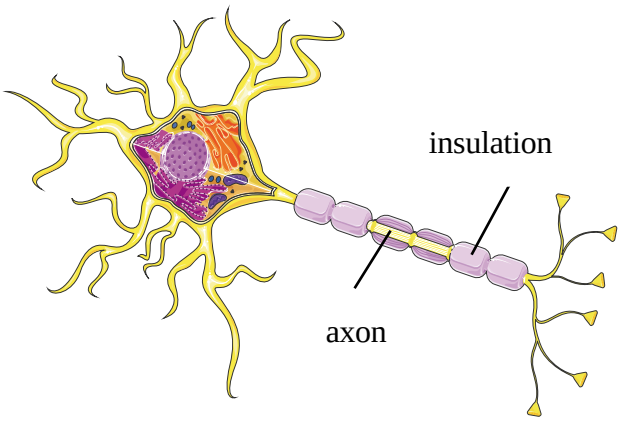

Figure 1. A neuron with an axon making connections to other neurons. Picture from Smart Servier Medical Art, https://smart.servier.com, CC BY 3.0.

One example of such signaling molecules is the *endocannabinoids*. These lipid molecules are called like this because they are made by the body itself (they are "endogenous") and they can bind to the same receptors as the drug cannabis (they are cannabis-like, "cannabinoid"). It is already known that the endocannabinoids and their receptors play a role in the early development of the brain, by helping the axons of neurons to grow and find their way.

In **study I**, we wanted to find out exactly how endocannabinoids influence the pathfinding of axons. When we increased the amount of an endocannabinoid in the developing mouse brain, we discovered mistakes in the pathfinding of growing axons and the faster development of another cell type in the brain called oligodendrocytes. These cells wrap insulation material

around the axons of neurons (see **figure 1)** so that information can travel faster to the next neuron. Having a too high amount of the endocannabinoid made the mature oligodendrocytes release extra Slit2, the molecule that can bind to the receptor Robo1, which at the same time moved to the growing ends of axons. The interaction of Robo1 and Slit2 gives a repulsive signal ("bad smell") to the growing ends of axons, which is why they turn away and make mistakes in their pathfinding. This finding contributes to our knowledge of the consequences of cannabis abuse by pregnant women for the brain of developing children.

Another group of signaling molecules is the so-called *neuropeptides*, super small proteins released from neurons that also bind to receptors on other neurons. We were the first to find in **study II** that one such a neuropeptide, called galanin, is present in the neurons of a specific mouse brain region only during the first two weeks after birth. This finding is interesting, because the development of the brain is not finished when a baby or a mouse pup is born but continues for weeks in mice to years in humans after birth.

In general, the neurons in the newborn brain make more connections than are strictly necessary. The additional connections are "trimmed", as if cutting back the branches of an overgrowing bush in your garden. The brain region where we discovered the temporary presence of galanin, called the somatosensory thalamus (see **figure 2**), is particularly fascinating in this regard, as it processes sensory information and helps to distinguish from which body part the signal exactly comes. The precision with which we can process sensory information depends on the right neurons being linked together, so the "trimming" of extra connections is essential. We think that galanin might be important for the development of the somatosensory thalamus after birth.

The same group of signaling molecules, the *neuropeptides*, are also found in many neurons in a different brain region, called the hypothalamus (see **figure 2**). This brain region is extremely important for our survival because it helps us to adapt to our ever-changing environment by steering processes such as sleep, food intake, stress, body temperature, aggression, and sex. Since the hypothalamus controls so many different functions, it is logical that the neurons here are remarkably diverse in their connections to other brain regions and in the molecules that they make. However, we don´t really know yet how molecularly diverse these neurons are, because we could until recently not study many cells at the same time in our experiments while also measuring many molecules in all these cells.

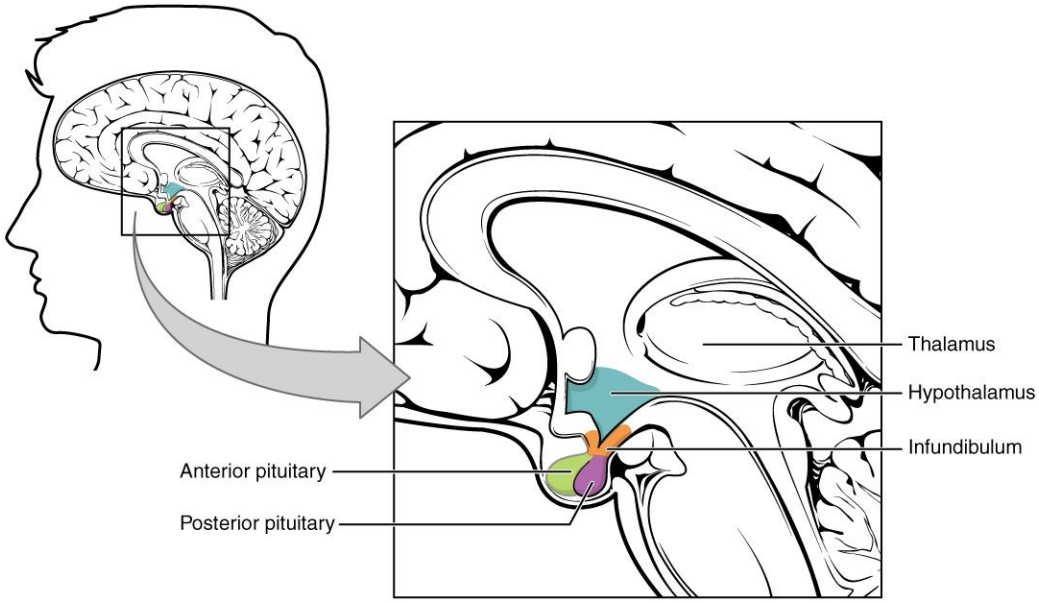

*Figure 2. Schematic of a human brain indicating the **thalamus**, the **hypothalamus** (blue) and the two parts of the **pituitary** (green and purple). The mouse brain looks similar. Picture from Anatomy & Physiology, Connexions website, OpenStax college. http://cnx.org/content/col11496/1.6/, CC BY 3.0.*

In **study III** we used a new technique called 'single-cell RNA-sequencing' to look at thousands of individual cells in the hypothalamus and classify these cells into different groups according to what genes they are expressing. The analysis of the hypothalamus in the mouse identified 62 neuronal subclasses, indicating a huge diversity of cells. The 62 groups of neurons can be most easily separated based on the signaling molecules they use, including the *neuropeptides* that were mentioned before.

Using a modified virus that we precisely injected into the hypothalamus, we marked the axons of one of these 62 groups of neurons to figure out to which other neurons they are connected. We found out that this new subgroup probably regulates the release of hormones from a structure called the pituitary (see **figure 2**) to the rest of the body. This new subgroup received inputs from neurons in the "pacemaker of the brain", indicating that it could be important for controlling the day-night rhythm of hormonal release. Since we did not only study this one subgroup but have information on all 62 groups of neurons, we created an online database where we shared the huge amount of data from this experiment with other scientists, who can look up their favorite neurons and find out which genes they express.

Finally, in **study IV**, we again studied the hypothalamus in the mouse, starting with some interesting results from the single-cell RNA-sequencing. We found that neurons in the hypothalamus that are known to respond to stress (by stimulating the release of stress hormones from the adrenal gland via the pituitary) also connect to the special cells on the wall of the "third ventricle" (see **figure 3**). The brain ventricles form a network of cavities filled with a special liquid that transports different molecules to different parts of the brain.

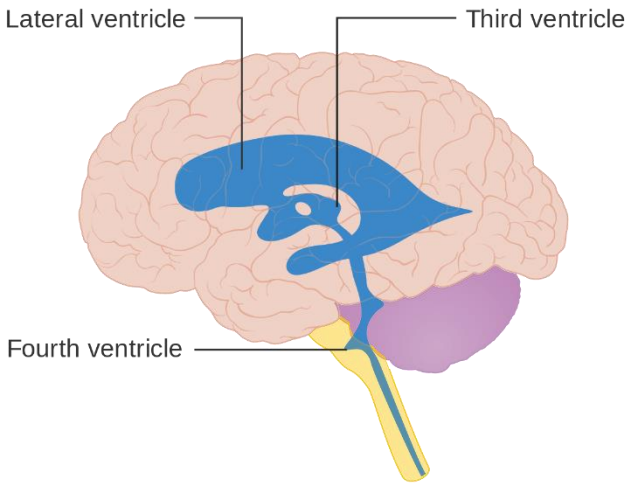

Figure 3. The network of the connected ventricles in the human brain. Picture from Cancer Research UK/Wikimedia Commons.

As said before, a neuron needs to make the receptor to be able to "listen" to a certain molecule. We discovered in study IV that in response to stress, the cells on the wall of the third ventricle release a molecule that travels all the way to receptors on neurons in a brain region lying next to the fourth ventricle. These neurons in turn make long connections to the front of the brain, where they activate the neurons that help us pay attention. You can imagine that this is extremely useful in stressful situations, but not if the stress becomes chronic. It is not healthy to always stay alert and "on": it is good to take a rest as well. We hope that the discovery of this new brain circuit might help to find treatments for chronic stress.

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